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Les récepteurs aux imidazolines dans le cœur : identification, distribution et fonction

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Résumé

Les récepteurs aux imidazolines I_1 sont des récepteurs neurotransmetteurs non adrénergiques, principalement trouvés dans la région rostro-ventrolatérale médullaire du tronc cérébral et dans les reins. Les récepteurs I_1 sont impliqués dans la régulation de la pression artérielle suite à leur activation par des imidazolines comme la moxonidine et la clonidine. La moxonidine, un composé imidazolinique antihypertenseur, réduit la pression artérielle par l'activation sélective des récepteurs I_1 et l'inhibition de l'activité du système nerveux sympathique, avec la moindre contribution des récepteurs α_2-adrénergiques. En plus, des études précédentes ont montré que le traitement aigu de la moxonidine induit la diurèse, la natriurèse et que le traitement chronique régresse l'hypertrophie ventriculaire gauche chez les patients et les animaux. Le but de ces études est de localiser et de caractériser les récepteurs I_1 et les récepteurs α_2-adrénergiques dans le cœur et de montrer que ces récepteurs sont fonctionnels. Nous avons montré que le traitement aigu à la moxonidine (i.v) des rats hypertendus (SHR) et des rats normotendus diminue la pression artérielle, stimule la diurèse, la natriurèse, la kaliurèse et stimule l'excrétion de GMPc de façon dose-dépendante. Ces effets sont associés avec l'augmentation du niveau plasmatique du peptide natriurétique auriculaire (ANP), qui est une hormone diurétique, natriurétique, vasodilatatrice et possède une activité sympatholytique et antihypertrophique. La contribution d'ANP a été confirmée par l'injection (i.v) de la moxonidine après le prétraitement des rats avec l'anantin, un antagoniste des peptides natriurétiques. Ces effets rénaux provoqués par la moxonidine étaient inhibés par l'anantin de façon dose-dépendante, indiquant l'implication de l'ANP dans les actions aiguës de la moxonidine. Ces effets sont aussi inhibés par l'efaroxan (antagoniste des récepteurs I_1) et par la yohimbine (antagoniste des récepteurs α_2-adrénergiques), indiquant l'implication des récepteurs I_1 et des récepteurs α_2-adrénergiques dans les actions aiguës de la moxonidine. Le traitement chronique (1e et 4e semaines) des SHR à la moxonidine résulte en une diminution dose-dépendante de la pression artérielle et une régression significative de l'hypertrophie ventriculaire gauche. Ces effets sont associés avec l'augmentation du niveau plasmatique et de l'expression auriculaire de l'ANP pendant la première semaine de traitement et la normalisation de ces paramètres

après 4 semaines de traitement à la moxonidine. Par conséquent, l'activation aiguë et chronique des récepteurs I_1 et des récepteurs α_2-adrénergiques résulte en une stimulation de la libération d'ANP. Comme l'ANP est principalement libéré des oreillettes, la présence des récepteurs I_1 et des récepteurs α_2-adrénergiques était explorée dans le cœur. L'identification de ces récepteurs a été accomplie par autoradiographie, sur les sections congelées du cœur et sur les membranes cardiaques, et par transfert Western sur les membranes cardiaques. Les études de liaison et du transfert Western ont révélé que seuls les récepteurs I_1 étaient régulés lors des désordres cardiovasculaires et suite au traitement chronique à un agoniste sélectif pour les récepteurs I_1. Par comparaison à l'espèce normale, les récepteurs I_1 ont été surexprimés de 2 fois plus, dans les oreillettes des SHR, dans les ventricules des hamsters cardiomyopathiques et dans les ventricules défaillants humains. En plus, la densité des récepteurs I_1 était normalisée durant la première semaine et après quatre semaines du traitement chronique à la moxonidine, sans aucun effet sur les récepteurs α_2-adrénergiques. Par la suite, la fonctionnalité des récepteurs I_1 était étudiée *in vitro* sur des cœurs isolés. Nous avons démontré que la perfusion des cœurs isolés et la stimulation des oreillettes isolées et les cardiomyocytes en culture par la moxonidine stimulent la libération d'ANP et que cet effet était plus prononcé chez les SHR. Ces études démontrent pour la première fois la présence des récepteurs I_1 et α_2-adrénergiques dans le cœur et que ces récepteurs sont fonctionnels vu qu'ils sont impliqués directement dans la libération de l'ANP. Donc, la moxonidine diminue la pression artérielle suite à l'activation des récepteurs I_1 dans le tronc cérébral et dans les reins et, par la suite, on peut ajouter un niveau mécanisme, par le biais de l'activation des récepteurs I_1 cardiaques et de leur capacité de libérer l'ANP, un régulateur important de la pression artérielle et du volume sanguin dans les conditions normales et pathophysiologiques. Les peptides natriurétiques peuvent contribuer aux mécanismes thérapeutiques des agonistes des récepteurs aux imidazolines via leurs effets sympatholytiques, antihypertrophiques et antifibrotique.

Mots-clés : récepteurs aux imidazolines, ANP, hypertension artérielle, hypertrophie ventriculaire gauche, récepteurs α_2-adrénergiques, anantin, moxonidine.

ABSTRACT

Imidazoline I_1-receptor is a non-adrenergic neurotransmitter receptor mainly found in the brainstem rostral ventro-lateral medulla and kidneys. I_1-receptors have been implicated in blood pressure regulation by imidazoline drugs such as moxonidine and clonidine. Moxonidine, an imidazoline antihypertensive compound, reduces blood pressure primarily by selective activation of central imidazoline I_1 receptors, with the contribution of α_2 adrenoceptors and inhibition of sympathetic nervous system (SNS) activity. In addition, previous studies have shown that acute moxonidine treatment induces diuresis and natriuresis and that chronic treatment results in regression of left ventricular hypertrophy in hypertensive patients and animals. The aim of these studies was to localize, caracterize and investigate functionality of these receptors. We have shown that acute iv administration of moxonidine to 12-14-week old normotensive and spontaneously hypertensive rats (SHR) decreased blood pressure, evoked diuresis, natriuresis, and kaliuresis and stimulated urinary cGMP excretion in a dose-dependent manner. These effects were associated with elevated circulating atrial natriuretic peptide (ANP) levels. ANP is a diuretic, natriuretic and vasodilator hormone, with sympatholytic and anti-hypertrophic activities. The implication of ANP in the acute renal effects of moxonidine was demonstrated in normotensive (SD and WKY) and SHR that received acute iv injections of moxonidine with or without anantin, an antagonist of natriuretic peptides. Pretreatment with anantin dose-dependently inhibited the urinary effects of moxonidine, thus implicating ANP in the acute action of moxonidine. The renal responses to moxonidine were also inhibited by efaroxan (I_1 receptors antagonist) and by yohimbine (α_2-adrenoceptor antagonist), implying that I_1-receptors and α_2-adrenoceptors are involved in the acute actions of moxonidine. In addition, chronic treatment of SHR with moxonidine during one and four weeks resulted in a dose-dependent decrease in blood pressure, and significant regression of left ventricular hypertrophy after 4 weeks of treatment. These effects were associated with increased plasma levels and atrial expression of ANP measures after one week of treatment, and normalization after four weeks of treatment.

Taken together, these studies implied that acute and chronic activation of imidazoline receptors (and α_2-adrenoceptors) results in stimulated ANP release. Therefore, since ANP is primarily secreted by cardiac atria, the presence of I_1-receptors and α_2-adrenoceptors was investigated in the heart. Identification of I_1-receptors was performed by autoradiography on frozen heart sections and on cardiac membranes, and by western blots in atrial and ventricular membranes. Binding studies and immunoblotting revealed that only heart I_1 receptors were regulated in cardiovascular diseases and in response to chronic in vivo exposure to a selective agonist. Quantification by membrane binding and autoradiography, revealed that relative to respective controls, I_1-receptors were up-regulated by 2 fold in atria of hypertensive rats and in ventricles of hamsters with advanced cardiomyopathy, and human ventricles with heart failure. In addition, only up-regulated I_1 receptors were normalized by short- and long-term *in vivo* treatment with moxonidine, without any effect on α_2-adrenoceptors. In addition, functionality of cardiac I_1-receptors was investigated *in vitro* without central contribution. In perfused isolated hearts, atria, and cardiomyocytes in culture, moxonidine stimulated ANP release and the effect was more pronounced in SHR. The present studies show for the first time that the heart exhibits both, imidazoline receptors and α_2-adrenoceptors and that these receptors are functional, since upon activation they stimulate the release of ANP. Moxonidine that has been shown to decrease blood pressure by selective activation of imidazoline receptors in the brain stem and action on the kidneys, may also reduce blood pressure through another mechanism namely activation of functional imidazoline receptors in the heart and subsequent release of ANP, which plays an integral role in volume and pressure homeostasis in normal and pathophysiological conditions. Implication of natriuretic peptide may also contribute to the therapeutic mechanism of imidazoline receptor agonists through their sympatholytic, anti-proliferative and anti-fibrotic effects.

Keywords: hypertension, imidazoline receptors, α_2-adrenoceptors, ANP, left ventricular hypertrophy, anantin, heart.

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ABRÉVIATIONS

^{125}I-PIC	radioiodinated *p*-iodoclonidine
1K1C	one kidney one clip
AMPc	adénosine 3',5'-monophosphate cyclique
ANP	peptide natriurétique auriculaire
AVP	vasopressine
BNP	peptide B-natriurétique
CNP	peptide natriurétique de type C
DAG	diacylglycérol
ERK	extracellular regulated protein kinase
GMPc	guanosine 3',5'-monophosphate cyclique
HVG	hypertrophie du ventricule gauche
iECA	inhibiteurs de l'enzyme de conversion
JNK	c-jun-N-terminal kinase
MAO	monoamine oxydase
MAPK	mitogen-activated protein kinase
NEP	endopeptidase neutre
NPR-A	récepteur des peptides natriurétiques de type A
PC12	adrenal pheochromocytoma cells
PC-PLC	phosphatidylcholine sélective phospholipase C
PKC	protéine kinase C
RVLM	région rostro-ventrolatérale medulla
SD	rat normotendu de type *Sprague Dawley*
SHR	rat spontanément hypertendu

SNC	système nerveux central
SNS	système nerveux sympathique
TNF-α	tumor necrosis factor-alpha
WKY	rat normotendu de type Wistar Kyoto

À mes parents et ma famille

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CHAPITRE 1

Introduction

1.1-HYPERTENSION ARTÉRIELLE

L'hypertension artérielle demeure un problème sérieux de santé publique au Canada et ailleurs dans le monde, affectant environ 25 % de la population canadienne. Elle constitue un facteur de risque important d'accidents vasculaires cérébraux, d'insuffisance cardiaque, d'insuffisance rénale et de maladies vasculaires périphériques, et est considérée comme la première cause de décès à l'échelle nationale (Joffres et coll., 2004).

1.1.1-Définition

La pression artérielle observée dans la circulation possèdent deux caractéristiques : (a) une pression moyenne et (b) une pression pulsatile. En effet, la pression moyenne est déterminée par le débit cardiaque et la résistance vasculaire, qui sont à leur tour influencés par le calibre et le nombre des petits artères et des artérioles. La composante pulsatile est caractérisée par la pression pulsée, qui est définie par l'oscillation de l'onde artérielle; la systole et la diastole répresentant le pic et le creux de l'oscillation, respectivement. Les niveaux de la pression pulsée est déterminé principalement par l'éjection ventriculaire gauche, les propriétés élastiques des artères de gros calibre ainsi que l'onde pulsatile en périphérie.

L'hypertension artérielle se définit par une augmentation de la pression artérielle de manière chronique. Environ 5 % des sujets hypertendus souffrent d'une forme d'hypertension dite secondaire dont la cause est identifiable ou secondaire à une pathologie sous-jacente (Onusko, 2003). Les pathologies rénales (rétrécissement

réno-vasculaire) et les pathologies endocriniennes (aldostéronisme primaire, phéochromocytome et maladies thyroïdiennes) peuvent être comprises dans l'hypertension secondaire. D'autre part, dans 95 % des cas, il n'existe pas de cause identifiable de l'hypertension désignée comme hypertension primaire ou idiopathique (Bjorntorp et coll., 2000). Les interactions entre les facteurs génétiques et environnementaux peuvent contribuer au développement de l'hypertension primaire en affectant les mécanismes impliqués dans cette forme, comme la fonction rénale, le système rénine-angiotensine-aldostérone, les catécholamines circulantes, l'endothéline et le stress oxydatif.

Plusieurs études ont permis de mieux apprécier la diversité des facteurs, à contribution primaire ou secondaire, pouvant être impliqués dans le développement de l'hypertension artérielle (Folkow, 1989). Plusieurs facteurs (génétiques, environnementale, anatomique, adaptative, neuronale, endocrinienne, humorale et hémodynamique) interagissent mutuellement pour contrôler la pression artérielle, suggérant qu'un déséquilibre entre ces différentes facteurs puisse produire une élévation de la pression artérielle.

Plusieurs substances endogènes et systèmes physiologiques influencent, de façon directe ou indirecte, la pression artérielle. Cette situation a contribué toujours à l'émergence de nouvelles hypothèses dans ce domaine de recherche très dynamiques. L'étiologie du développement de l'hypertension artérielle est encore mal définie. Le système nerveux sympathique le système rénal et le système rénine angiotensine, parmi les facteurs hormonaux, font toujours l'objet d'importantes recherches afin d'évaluer, avec une plus grande précision, leur contribution au développement et au maintien de l'hypertension artérielle.

Un facteur commun qui influence tous les mécanismes ci-dessus est le système nerveux sympathique (SNS). En conséquence, le SNS joue un rôle important dans la régulation de la pression artérielle et de la fonction cardiaque.

L'activité nerveuse sympathique est régulée dans les centres du cortex cérébral et transmise aux organes et aux vaisseaux sanguins qui sont innervés par les terminaisons nerveuses sympathiques. L'activation de l'efflux sympathique au cœur, aux reins et aux vaisseaux du muscle squelettique est généralement observée chez les jeunes patients présentant de l'hypertension essentielle. Les indices d'une activation de SNS, tels que l'activité sympathique, la libération (ou *spillover*) de la norépinéphrine au niveau des organes cibles et les taux plasmatiques de la norépinéphrine sont augmentés de manière constante et significative chez les personnes souffrant d'hypertension essentielle par rapport aux personnes normotendues (Wallin et Sundlof, 1979; Esler et Kaye, 1998; DeQuattrov et Feng, 2002). Cette activité conduit à des risques cardiaques, comme le développement de l'hypertrophie ventriculaire gauche (HVG), prédisposant à l'arythmie ventriculaire, à la résistance d'insuline croissante et accélère l'athérogénèse. Le niveau d'excitation sympathique au cœur est une cause déterminante importante de pronostic chez les patients hypertendus et ceux présentant une défaillance cardiaque, et une diminution de la fréquence cardiaque et de l'activité du nerf sympathique pourrait être bénéfique dans le pronostic à long terme chez ces patients (Wenzel et coll., 2000).

Le SNS contribue au contrôle de la pression artérielle dans des conditions variables, en modifiant le débit cardiaque, la résistance vasculaire périphérique et la fonction rénale. Le SNS peut exercer des actions hypertensives puissantes et participe à la pathophysiologie de l'hypertension artérielle chronique par ses effets sur les reins.

Le rein, par ses qualités d'organe régulateur de la balance hydrosodée, est impliqué dans le contrôle de la pression artérielle et peut potentiellement contribuer au développement de l'hypertension artérielle (Guyton, 1992). En effet, le volume extracellulaire (volume plasmatique et volume interstitiel) est tributaire de la qualité de sodium dans l'organisme, le sodium étant le principal ion extracellulaire. Une augmentation de la réabsorption de sodium, déterminée génétiquement, pourrait effectivement conduire à une augmentation soutenue de

la pression artérielle (Bianchi et coll., 1989). Il ne faut pas oublier que cette fonction du rein est elle-même contrôlée et modulée par plusieurs facteurs neuronaux (dont le système nerveux sympathique), hormonaux et hémodynamiques qui pourraient, eux aussi, contribuer à induire une augmentation de la rétention du sodium. L'activation des nerfs sympathiques aux reins augmente la réabsorption tubulaire de sodium, la libération de rénine et la résistance vasculaire rénale (DiBona et Kopp, 1997) via l'activation de récepteurs α et β-adrénergiques, et indirectement en stimulant le système rénine-angiotensine par l'activation des récepteurs β_1-adrénergiques (Brodde et Michel, 1999). Ces actions contribuent à l'élévation de la pression artérielle à long terme en décalant la courbe pression-natriurèse vers la droite, pour permettre au corps de se débarrasser de la charge sodique.

Le système de contrôle volume/pression rénal est considéré comme un facteur dominant dans la régulation physiologique de la pression artérielle à long terme, en raison de ses possibilités infinies de contrôler l'excrétion d'eau et des électrolytes en réponse à l'augmentation ou la diminution de la pression artérielle systémique (Guyton, 1990).

Les effets hypertensinogéniques du SNS sur le rein peuvent commencer dès l'ontogenèse. Chez les rats spontanément hypertendus (SHR), l'innervation sympathique rénale est augmentée (Gattone et coll., 1990) et la sympathectomie néonatale est associée à une diminution de la pression artérielle à long terme. Les reins des SHR néonataux, sympathectomisés transplantés chez les destinataires non traités, contribuent à une diminution de la pression artérielle d'environ 20 mm Hg à long terme et à une réduction de la sensibilité de la pression artérielle au sodium, suggérant que l'innervation sympathique néonatale des SHR provoque des changements de la fonction rénale qui sont impliqués dans le développement et la maintenance de l'hypertension (Churchill et coll., 2001; Grisk et coll., 1999).

Le SNS et le système rénine-angiotensine constituent les deux principaux systèmes de régulation de la pression artérielle. Ces systèmes ont la capacité de

s'influencer mutuellement. Ainsi, une activation des récepteurs β_1-adrénergiques stimule la libération rénale de rénine, ayant pour conséquence d'augmenter la quantité circulante d'angiotensine II. L'action principale de l'angiotensine II au niveau du système circulatoire est une réponse pressive compensatoire aiguë suite à une diminution du volume plasmatique. Cette réponse pressive est causée par une vasoconstriction permettant de réduire la capacité du lit artériel (augmentation de la résistance artérielle) et par une libération accrue de l'aldostérone permettant de retenir le sodium. Tout d'abord, l'angiotensine II pourra moduler l'activité sympathique à plusieurs niveaux et entraînerait une augmentation de la production de catécholamines au niveau des noyaux du système nerveux central. L'angiotensine II pourrait également augmenter la quantité de norépinéphrine et d'épinéphrine libérées lors de chaque influx nerveux au niveau de la terminaison nerveuse présynaptique (Zimmerman, 1981). Ensuite, l'angiotensine II posséderait la capacité de faciliter la mobilisation du calcium intracellulaire au niveau des neurones sympathiques (Fernandez et coll., 2003). Finalement, l'Ang II interagit avec les neurotransmetteurs du SNS pour favoriser la rétention d'eau et de sel (Ichihara et coll., 1997). Les interactions biologiques et fonctionnelles entre ces deux systèmes existent puisque l'angiotensine II et la NE peuvent induire toute une élévation de la pression artérielle ainsi qu'une croissance des cellules du muscle lisse vasculaire. Dans une perspective globale, ces données nous indiquent que le système rénine-angiotensine est un régulateur important de la pression artérielle.

1.1.2-**Traitement de l'hypertension**

L'objectif thérapeutique de l'hypertension artérielle est non seulement de diminuer la pression artérielle, mais aussi de prévenir le risque de complications cardiovasculaires. En raison de la complexité de l'hypertension artérielle, son caractère multifactoriel et sa composante génétique importante, il est évident qu'il n'existe pas un traitement unique et universel pour tous les hypertendus. Le traitement inclut des approches pharmacologiques et non pharmacologiques.

Le changement de mode de vie et l'augmentation de l'activité physique pour stimuler la perte du poids sont des thérapies primaires non pharmacologiques.

Cependant, la thérapie pharmacologique est orientée pour réduire la vasoconstriction et la rétention du sodium par l'utilisation d'un agent ou la combinaison de plusieurs agents antihypertenseurs (Figure 1).

Les agents couramment utilisés dans le traitement de l'hypertension incluent les diurétiques (les thiazides) qui sont divisés en trois catégories : les thiazides (chlorthalidone), les diurétiques de l'anse (furosémide) et les diurétiques épargneurs de potassium (spironolactone). Ils agissent au niveau des tubules rénaux pour provoquer la diminution de la volémie et de la surcharge sodique de l'organisme, en stimulant l'élimination de l'eau et de sodium (Na^+) par le rein (Lang, 1987; Wittner et coll., 1991). Les vasodilatateurs (l'hydralazine) diminuent directement la pression sanguine par la dilatation des vaisseaux périphériques. Ils peuvent agir sur les vaisseaux de résistance, les grosses artères et les vaisseaux de capacitance. Certaines conséquences négatives de la vasodilatation directe limitent l'utilisation de ces drogues en monothérapie. La dilatation artérielle directe déclenche une activation sympathique médié par les barorécepteurs, résultant en une tachycardie et une augmentation du débit cardiaque, donc ils peuvent être utilisé seulement en combinaison avec des drogues sympatholytiques. Les antagonistes des canaux calciques sont efficaces dans le traitement de l'hypertension par leurs effets vasodilatateurs périphériques, médié par le blocage des canaux calciques de type L qui répresente la voie majeure du calcium dans le muscle lisse vasculaire suite à la dépolarisation (Sun et coll., 2000; Noll et coll.. 1998). Ils réduisent l'influx de calcium, la contraction de muscle lisse et la conductivité éléctrique. Sur la base de la structure chimique, trois groupes majeurs d'antagonistes calciques peuvent être distingués : la classe des phénylalkylamines (verapamil), des benzothiazépines (diltiazem) et des dihydropyridines (nifedipine) (Fleckenstein, 1990).

Les inhibiteurs de l'enzyme de conversion de l'angiotensine (iECA), comme le captopril, inhibent la production de l'angiotensine II de l'angiotensine I. La réduction des niveaux d'agiotensine II circulants mène à une diminution des effets du peptide sur le tonus vasculaire (Zimmerman et coll., 1984), sur l'expansion volumique à travers la rétention sodique par le biais de l'aldostérone (Contreras et coll., 2003) et sur la rétention de fluide par la vasopressine (AVP) (Padfield et Morton, 1977). En plus, les iECA interfèrent avec la transmission sympathique en antagonisant l'action facilitatrice de l'angiotensine II sur le relâchement des transmetteurs présynaptiques (Saxena, 1992). Des effets semblables sont réalisés en utilisant des antagonistes de récepteur de l'angiotensine AT1, comme le losartan et le candésartan (Timmermans et coll., 1993; Martineau et Goulet, 2001).

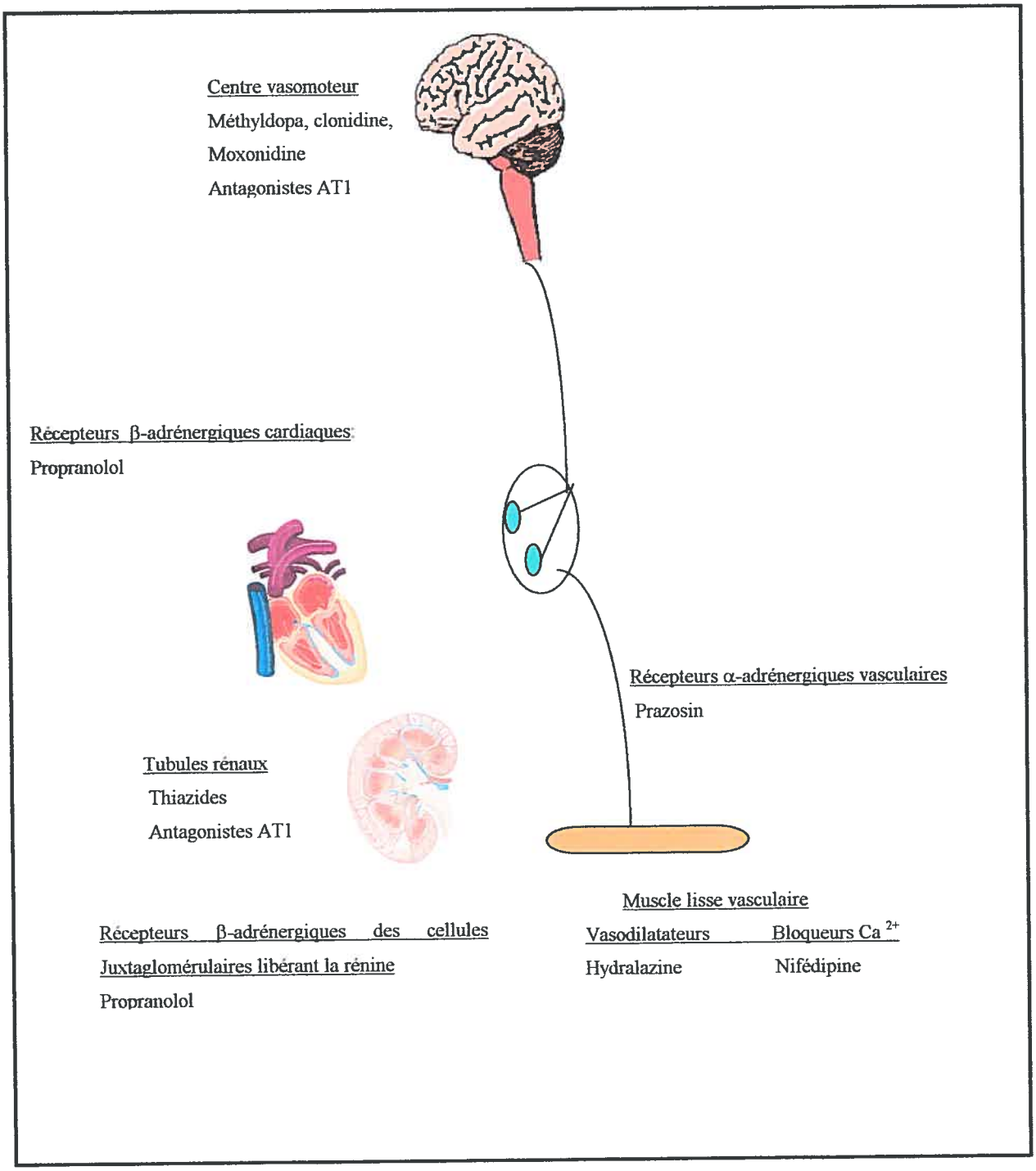
D'autres traitements antihypertenseurs incluent l'inhibition directe de l'activité de SNS. Cette inhibition est réalisée par plusieurs drogues agissant à plusieurs étapes depuis la synthèse des neurotransmetteurs jusqu'à leurs actions sur leurs récepteurs post-synaptiques. Les bloqueurs des récepteurs α_1-adrénergiques, tels que la prazosine, bloquent la liaison de la norépinéphrine aux récepteurs α_1-adrénergiques en diminuant la résistance vasculaire périphérique et par la suite la pression artérielle (Cubeddu, 1988; Luccioni et coll., 1990). Les béta-bloqueurs inhibent les effets de la stimulation sympathique au niveau cardiaque. Les béta bloqueurs diminuent la fréquence cardiaque (effet chronotrope négatif), la contractilité myocardique (effet inotrope négatif), et le débit cardiaque (Schafers et coll., 1994). Les béta bloqueurs diminuent la production de rénine par l'appareil juxtaglomérulaire (cette action participe à l'effet anti-hypertenseur). L'effet anti-hypertenseur dépend du blocage des récepteurs β_1-adrénergiques.

Une autre approche est l'utilisation des drogues qui empêchent la libération de la norépinéphrine. De telles drogues incluent les agonistes des récepteurs α_2-adrénergiques centraux, dont la clonidine et l'α-méthyldopa (MacDonald et coll., 1988) (Figure 1). Ces agonistes centraux inhibent l'activité du SNS et diminuent

la libération des catécholamines des terminaisons nerveuses suite à la stimulation des récepteurs α_2-adrénergiques présynaptiques et par conséquent conduit à une diminution de la pression sanguine (Wenzel et coll., 2000). Malgré leur efficacité d'antihypertenseurs, les agonistes des récepteurs α_2-adrénergiques ne sont pas utilisés comme des drogues de premier choix à cause de leurs effets secondaires, comme la sédation, la bouche sèche et la dépression mentale (Engelman, 1988). Les études avancées sur le contrôle de la pression artérielle continuent à donner de nouveaux agents qui peuvent être utilisés en thérapie, comme la moxonidine (Armah et coll., 1988; Ernsberger et coll., 1994) et la rilménidine qui diminuent la pression artérielle en inhibant l'activité de SNS (Safar, 1989). La moxonidine (Physiotens) est un antihypertenseur du groupe des agonistes des récepteurs aux imidazolines. Son effet au niveau du système nerveux central entraîne une diminution de l'activité sympathique périphérique, ce qui conduit à une diminution de la résistance vasculaire périphérique et, conjointement, à une baisse de la pression artérielle. Cette drogue est approuvée et utilisée en Europe depuis 1991. L'évaluation de la moxonidine dans les essais contrôlés a démontré que la moxonidine était aussi efficace que les autres principales classes d'antihypertenseurs tels le captoril, la nifedipine, l'aténolol ou l'hydrochlorothiazide (Küppers et coll., 1997 ; Kraft et Vetter, 1994 ; Bousquet et Feldman, 1999). Cette efficacité de la moxonidine est basée sur le fait qu'elle diminue la pression artérielle d'une façon comparable aux autres agents antihypertenseurs. De plus, elle était très bien tolérée chez les patients, l'arrêt du traitement n'a pas entraîné d'augmentation de la pression artérielle et les effets secondaires de la moxonidine sont mineurs (9 % des patients ont mentionné le problème de la bouche sèche tandis que 7% se sont plaints de troubles du sommeil). Seule l'étude MOXCON, conduite en 1999, a présenté des résultats non satisfaisants concernant le traitement des patients avec insuffisance cardiaque (NYHA, class II-IV) à la moxonidine. Cette étude a montré que la thérapie avec la moxonidine était associée à une augmentation de la mortalité et de la morbidité à court terme, ce qui amené l'arrêt prématuré de cette étude. Ces résultats de

MOXCON seraient dus au fait que cette étude n'était pas bien planifiée (Coats, 1999).

Les agents imidazoliniques (moxonidine et rilménidine) qui possèdent une grande sélectivité pour les récepteurs aux imidazolines que pour les récepteurs α_2-adrénergiques, possèdent moins d'effets secondaires qui sont attribués aux récepteurs α_2-adrénergiques (Armah et coll., 1988). Ceci donne une lueur d'espoir pour les agents plus sélectifs **des récepteurs aux imidazolines,** qui possèdent moins d'effets secondaires afin de contrôler la pression artérielle et rétablit un grand intérêt pour cette classe de drogues antihypertenseurs.



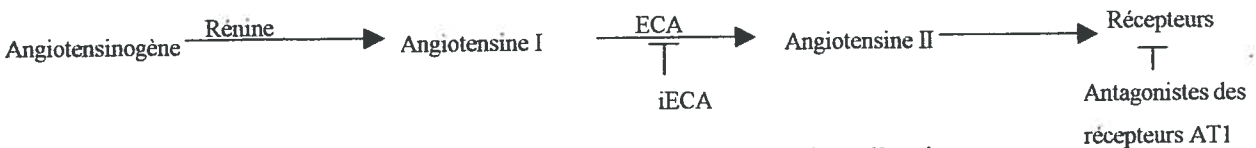


Figure 1: Les principaux agents antihypertensifs et leurs sites d'action,

(d'après le site: www.*fleshandbones.com*).

1.2-Les récepteurs aux imidazolines

1.2.1-Découverte

Le concept des récepteurs aux imidazolines a été proposé en se basant sur des études de recherche de sites d'action de la clonidine dans le cerveau, connue à cette époque comme un agoniste des récepteurs α_2-adrénergiques. Bousquet et coll., (1984) avaient démontré que l'administration de la clonidine directement dans la région rostro-ventrolatérale médullaire (RVLM), région connue comme modulatrice de la pression artérielle, induisait une diminution de la pression artérielle chez les chats anesthésiés (Bousquet et coll., 1984). Cet effet hypotenseur n'était pas observé avec les catécholamines ou les phényléthylamine, incluant l'α-méthyle noradrénaline, l'agoniste le plus sélectif pour les récepteurs α_2-adrénergiques, injecté dans la même région. Cependant, des études ont démontré que, parmi les antagonistes des récepteurs α_2-adrénergiques, seuls les antagonistes imidazoliniques préviennent l'effet hypotenseur des composés imidazoliniques (qui possèdent un anneau de cinq membres contenant deux nitrogènes) administrés dans la même région (Bousquet et coll., 1984). Il a été démontré par la suite qu'aucune corrélation n'était établie entre l'affinité des imidazolines pour les récepteurs α_2-adrénergiques et la diminution de la pression artérielle qu'elles induisent lors de leur injection directe dans cette région (Ernsberger et coll., 1988). Ceci indique que l'effet hypotenseur de la clonidine et de ses analogues n'est pas médié par les récepteurs α_2-adrénergiques, mais par des récepteurs non adrénergiques préférant les composés imidazoliniques (Figure 2).

Cette proposition a été confirmée par les études de liaison sur les membranes de RVLM qui ont démontré la présence des sites de liaison non adrénergiques, spécifiques pour les imidazolines (Ernsberger et coll., 1988). Ces sites possèdent une faible affinité pour les catécholamines et une forte affinité pour les substances imidazolinique (Bricca et coll., 1989; Ernsberger et coll., 1994). Il y avait une

corrélation entre l'affinité des substances imidazoliniques et la diminution de la pression lors de l'injection dans la même région.

Les récepteurs non adrénergiques, préférant les composés imidazoliniques, sont des entités moléculaires différentes et non pas un nouveau sous-type de récepteurs α_2-adrénergiques (Bousquet et coll., 1984; Schlicker et coll., 1997). Les récepteurs aux imidazolines reconnaissent la majorité, mais pas tous les imidazolines, et lient encore d'autres composés comme les oxazolines et certains imidazoles (Figure 2) (Bricca et coll., 1989; Ernsberger et coll., 1994).

Les études de liaison ont démontré au moins deux populations des sites aux imidazolines (Michel et Insel, 1989) : le sous-type-I_1, qui est impliqué dans la régulation de la pression artérielle, a plus d'affinité pour le [^{3}H] clonidine et ses analogues (Ernsberger et coll., 1988; Bricca et coll., 1993) tandis que le sous-type I_2 possède plus d'affinité pour le [^{3}H] idazoxan (Tesson et coll., 1991). Le groupe de Parini (1996) a démontré que la protéine I_2 purifiée partage des séquences communes des acides aminés avec l'enzyme monoamine oxydase (MAO); cette enzyme est impliquée dans la biodégradation des monoamines aromatiques, incluant les neurotransmetteurs, comme la sérotonine, l'épinéphrine, l'histamine et la dopamine, et joue un rôle dans des désordres psychiatriques et neurologiques (Parini et coll., 1996; Zhu et Piletz, 2003). Ces séquences sont caractérisées comme des sous-unités régulatrices de MAO (Raasch et coll., 1999) et, par conséquent, n'ont pas fait l'objet de notre étude. En outre, d'autres sous-types des récepteurs aux imidazolines, les récepteurs non-I_1, non-I_2, possédant des propriétés pharmacologiques différentes des récepteurs I_1 et I_2, sont caractérisés sur le versant présynaptique des tissus cardiovasculaires (Moldering et Gothert 1999; Gothert et coll., 1999). Ces récepteurs inhibent la libération de la norépinéphrine de l'artère pulmonaire de lapin (Gothert et Moldering, 1991) et des cœurs isolés de lapin, suite à l'activation des récepteurs aux imidazolines par la moxonidine, (Fuder et Schwarz, 1993). Récemment, ils ont démontré que les sites I_3 sont impliqués dans la libération de l'insuline des cellules β-pancréatiques (Morgan et Chan, 2001).

Les sites de liaison I_1 sont considérés comme des récepteurs, car ils répondent à tous les critères des récepteurs, incluant la spécificité de la liaison, l'association avec des fonctions physiologiques, la corrélation de l'affinité avec la fonction, la régulation physiologique, la possession des ligands endogènes, le couplage de ces récepteurs et, finalement, la solubilisation, la purification et la reconstitution (Roeske, 1984; Ernsberger, 1999). Les études de liaison avec les analogues de la clonidine radiomarqués sur le RVLM démontrent que cette liaison aux sites I_1 était spécifique, rapide, saturable, réversible et à haute affinité (Ernsberger, 1999). En plus, ces sites étaient reliés aux réponses physiologiques; des études menées avec des antagonistes sélectifs ont permis de distinguer formellement les actions pharmacologiques respectivement responsables des effets hypotenseurs des imidazolines et de leurs analogues structuraux.

Par conséquent, notre étude était centrée sur les sites I_1 qui sont considérés comme des récepteurs et sont impliqués dans la régulation de la pression artérielle.

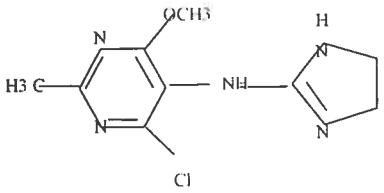
1.2.2-**Les récepteurs aux imidazolines I_1**

1.2.2.1-**Distribution et localisation cellulaires**

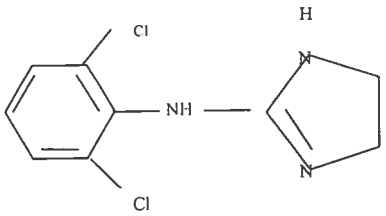
En utilisant [^{3}H] *p*-aminoclonidine, [^{3}H] clonidine et [^{125}I] *p*-iodoclonidine (Ernsberger et coll., 1990), les récepteurs aux imidazolines I_1 sont identifiés dans le cerveau, plus concentrés dans la RVLM, dans l'hippocampe et l'amygdale (Bricca et coll., 1989; Escriba et coll., 1994). Ces récepteurs sont aussi localisés dans les médullosurrénales de bovin (Ernsberger et coll., 1995), dans l'œil (Campell et Potter, 1994) et dans la prostate des humains et de chien (Felsen et coll., 1994). Les récepteurs aux imidazolines I_1 sont présents dans les reins, plus concentrés dans les tubules proximaux; leur densité diminue tout le long du néphron du mésangium jusqu'aux tubules collecteurs (Bidet et coll., 1990; Limon et coll., 1992; Bohmann et coll., 1994; Greven et von Bronewski-Schwarzer, 2001).

Au niveau cellulaire, les récepteurs I_1 dans le cerveau ne sont pas présents dans les astrocytes (Ernsberger et coll., 1990), mais ils sont localisés sur les membranes plasmiques des neurones du système nerveux (Heemskerk et coll., 1998; Ruggiero et coll., 1998). Les récepteurs I_1 sont présents dans les cellules chromaffines des glandes surrénales (Moldering et coll., 1993), les cellules phéochromocytomes (PC12) (Ernsberger et coll., 1995) et sur le glomus carotidien (Ernsberger et coll., 1998), dérivé embryogéniquement de la crête neurale. Les plaquettes (Piletz et Sletten, 1993) et les cellules épithéliales de la trachée (Liedtke et Ernsberger, 1995) expriment pareillement les récepteurs I_1. Ainsi, les récepteurs I_1 sont exprimés sur les cellules neuronales, épithéliales et sécrétoires.

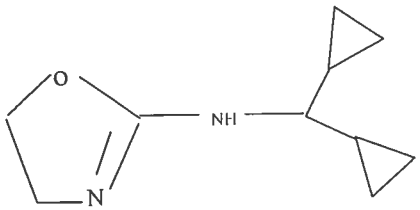
1-Moxonidine:4-chloro-5-(imidazolidin-2-ylidenimino)-6-methoxy-2-methylpyrimidine) (agoniste sélectif pour les récepteurs I_1)



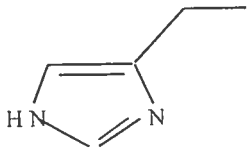
2-Clonidine: (2-[(2,6-dichlorophenyl)imino] imidazolidine monohydrochloride) (agoniste des récepteurs I_1 et α_2-adrénergiques)



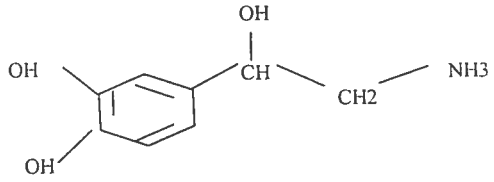
3-Rilménidine:[(Dicyclopropylmethyl)amino]-2delta2-oxazoline dihydrogenophosphate (agoniste sélectif pour les récepteurs I_1)



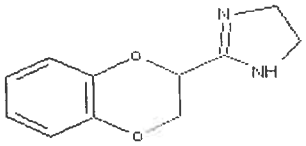
4-Imidazole-4 acide acétique (IAA) (agoniste pour les récepteurs I_1)



5-Norépinéphrine (agoniste des récepteurs α_2-adrénergiques)



6-Idazoxan: (antagoniste pour les récepteurs I_2/α_2-adrénergiques)



7-Efaroxan: 2-(2-ethyl-2,3-dihydro-2-benzofuranyl) -2-imidazoline (antagoniste sélectif pour récepteurs $I_1>\alpha_2$-adrénergiques)

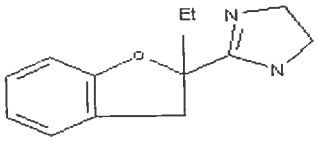


Figure 2 : les agonistes et les antagonistes des récepteurs I_1 et α_2-adrénergiques et la différence de structure entre les composés.

1.2.2.2-**Différences entre les récepteurs I_1 et α_2-adrénergiques**

Les récepteurs aux imidazolines sont différents des récepteurs α_2-adrénergiques et des récepteurs histaminiques (Bousquet et coll., 1984; Schlicker et coll., 1997). Les sites aux imidazolines peuvent être solubilisés et séparés physiquement des récepteurs α_2-adrénergiques et plusieurs équipes de chercheurs ont tenté de purifier ces sites de liaison et ont démontré qu'ils sont constitués d'entités protéiques différentes de celles de récepteurs α_2-adrénergiques (Parini et coll., 1996), démontrant que les sites α_2-adrénergiques et les sites aux imidazolines sont des entités différentes. Plusieurs caractéristiques différencient très nettement les récepteurs aux imidazolines des récepteurs α_2-adrénergiques, auxquels était initialement attribué l'effet hypotenseur des analogues de la clonidine. Il s'agit notamment de l'insensibilité des récepteurs I_1 aux analogues du GTP, indiquant l'absence de couplage à une protéine G, tandis que les récepteurs α_2-adrénergiques sont manifestement couplés à une protéine G (Bricca et coll., 1994). Par comparaison aux sites α_2-adrénergiques, les récepteurs I_1 sont insensibles au changement de la concentration sodique et des cations divalents et ses analogues et sont sensibles aux ions K^+ (Zonnenchein et coll., 1990, Lachaud-Pettiti et coll., 1991).

1.2.2.3-**Identification moléculaire des récepteurs I**

Depuis la mise en évidence de l'existence des récepteurs aux imidazolines dans le cerveau, de nombreuses études ont été réalisées dans le but de caractériser et d'isoler les protéines correspondantes à ces récepteurs. Plusieurs protéines qui correspondent aux récepteurs I ont été identifiées grâce aux deux anticorps (Wang et coll., 1993; Bennai et coll., 1996). Les protéines immunoréactives de différents poids moléculaires ont été détectées dans le cerveau de rat (45kDa) (Escriba et coll., 1995), le cerveau humain (43 kDa) (Bennai et coll., 1996; Greney et coll., 1994), les cellules adrénomédullaires de bovin (31 kDa, 70 kDa, 90 kDa) (Wang et coll., 1993; Ivanov et coll., 1998), les plaquettes humaines (33 kDa, 95 kda) (Ivanov et coll., 1998) et

dans les cellules mégakaryoblastomes humaines (MEG-01) (33 kDA et 85 kDa) (Ivanov et coll., 1998).

La protéine de 85 kDa a été corrélée avec la densité de sites de liaison et était considérée comme une candidate pour les récepteurs I_1 dans les cellules MEG-01. Il était suggéré que les protéines de faible poids moléculaire observées dans les études précédentes ne soient que des fragments protéolytiques de la protéine 85 kDa.

Plusieurs tentatives de clonage, en utilisant deux anticorps formés contre les protéines des récepteurs aux imidazolines (IRP) (Wang et coll., 1993; Bennai et coll., 1996), ont été réalisées récemmemt et ont permis de mettre en évidence quelques protéines potentielles des récepteurs aux imidazolines. Piletz et coll., (2003) ont réussi à isoler un cDNA à partir d'une banque de cDNA d'hippocampe humain qui possède des caractéristiques pharmacologiques se rapportant aux récepteurs I_1 (Ivanov et coll., 1998). Cet cDNA, isolé et amplifié, code pour la protéine de 167 kDa (1504 acides aminés) qui a été nommé IRAS pour Imdazoline Receptor Antisera-Selected. Cette protéine (IRAS-1) ne possède pas d'homologie avec les récepteurs α_2-adrénergiques et les monoamines oxydases et retient les propriétés de sites de hautes affinités pour la moxonidine et la rilménidine. Néanmoins, une série de trois domaines a été identifiée dans le IRAS-1 : un domaine riche en sérine, un domaine acide et un domaine riche en proline, ces motifs d'IRAS-1 paraissent similaires aux motifs des récepteurs de ryanodine et les récepteurs de cytokine (Piletz et coll., 2003). La transfection de IRAS-1 dans les cellules "Chinese hamster ovary cells" (CHO) aboutit à une liaison à haute affinité pour les récepteurs I_1 (Piletz et coll., 2003). L'IRAS-1 code pour le domaine fonctionnel des récepteurs aux imidazolines et pour la sous-unité intégrine $alpha_5$. Cette sous-unité joue un rôle dans la régulation de la croissance et de la survie de certains types cellulaires (Sastry et coll. 1999; Zhang et coll. 1995; O'Brien et coll. 1996; Lee et Juliano, 2000). Une étude récente a montré que cette protéine possède 80% d'homologie avec la Nischarine, qui est l'homologue d'IRAS chez la souris (Alahari et coll., 2004). Cette protéine interagisse avec le

récepteur à la fibronectine et inhibant la migration cellulaire (Alahari et coll., 2000). Il est donc nécessaire de continuer les travaux de recherche dans ce domaine pour savoir le lien existant entre ces protéines et le récepteur I_1.

1.2.2.4-**Ligands endogènes**

Atlas et Burstein (1984) et Atlas et coll., (1987) ont purifié, des extraits du cerveau de rat et de veau, une substance qui possède la capacité d'inhiber la liaison de [^{3}H] clonidine sur les membranes de RVLM et pour cette raison elle était nommée "clonidine displacing substance" (CDS). Par la suite, grâce aux études de liaison, le CDS (588 kDa) était montré plus sélectif pour les récepteurs aux imidazolines (Piletz et coll., 1995) que les récepteurs α_2-adrénergiques (Atlas et Burstein, 1984). Le CDS est distribué dans les tissus centraux et périphériques, il a été détecté dans le cerveau de plusieurs espèces (Atlas et Burstein, 1984; Meeley et coll., 1986), dans le sérum humain (Synetos et coll., 1991), dans le placenta (Diamant et coll., 1992) et dans les cellules gliales (Reis et Regunathan, 1995).

Li et coll. (1994) ont caractérisé et purifié l'agmatine (130 kDa) comme un candidat de CDS. L'agmatine a été identifiée chez les plantes et les bactéries (Tabor et Tabor, 1984), mais n'a jamais été identifiée chez les mammifères. L'agmatine s'est révélée présente dans presque tous les organes de mammifères et de rats, dans l'estomac, l'aorte, les intestins et la rate, les poumons, les surrénales, les reins, le cœur, le foie, le plasma, les cellules endothéliales et le muscle squelettique (Raasch et coll., 1995; 2002). L'agmatine, un précurseur des polyamines (Sun et coll., 1995), est synthétisée dans le cerveau à partir d'arginine en présence de l'enzyme spécifique, l'arginine décarboxylase (ADC) (Li et coll., 1995); par la suite, l'agmatine est stockée dans les neurones et libérée de façon calcium dépendante (Raasch et coll., 2001). Une fois libérée, l'agmatine agit sur les récepteurs aux imidazolines et α_2-adrénergiques pour médier des effets différents, comme la diminution de la pression artérielle (Raasch et coll., 2002). La diminution de la pression artérielle par l'agmatine était observée uniquement après l'injection intraveineuse; par contre, l'injection dans la région

centrale RVLM n'avait aucun effet sur la diminution de la pression artérielle, donc cet effet est médié en partie par vasodilatation (Regunathan et coll., 1996; 1999).

D'autres ligands endogènes ont été découverts chez les mammifères, incluant l'humain, comme l'harmane et le propyl harmane (β-carboline) qui ont été isolés dans les poumons de bovin (Parker et coll., 2004). Ces ligands permettent la relaxation des aortes de façon endothélium dépendant (Musgrave et coll., 2003) et possèdent une affinité nanomolaire pour les récepteurs I_1 et I_2 et pas pour les récepteurs α_2-adrénergiques. Contrairement à l'agmatine, la microinjection d'harmane dans la RVLM chez les rats provoque une diminution de la pression sanguine (Musgrave et Badoer, 2000). En raison de sa sélectivité pour les récepteurs I_1 par rapport aus récepteurs α_2-adrénergiques et de son effet pharmacologique sur les récepteurs I_1, l'harmane peut être le ligand endogène des récepteurs I_1. Récemment, l'acide imidazole-acétique ribotide (l'IAA-RP) était présenté comme un autre ligand des récepteurs I_1, identifié dans le cerveau et dans les neurones du cortex cérébral, incluant le RVLM (Prell et coll., 2004).

1.2.2.5-**Voies de signalisation des récepteurs I_1**

Les cellules phéochromocytomes (PC12), une lignée de cellules cancéreuses isolées de la glande surrénale, ont été utilisées pour étudier les mécanismes de la signalisation des récepteurs aux imidazolines. Le choix de ces cellules était basé sur le fait qu'elles expriment les récepteurs aux imidazolines I_1 et non les récepteurs α_2-adrénergiques (Separovic et coll., 1996). Il a été démontré que les récepteurs I_1 sont couplés à la phosphatidylcholine sélective phospholipase C (PC-PLC); suite à son activation, la PC-PLC utilise la phosphatidylcholine comme un substrat et génère le diacylglycérol (DAG) et le phosphocholine (Separovic et coll., 1996). Le DAG, sous l'action du DAG lipase, permet la libération de l'acide arachidonique (Figure 3) (Ernsberger, 1999). En plus, le DAG active deux types de protéine kinase C (cPKCβ_{II} et a PKCξ) suite à l'activation des récepteurs I_1. La PKC phosphoryle la famille de "*mitogen-activated protein kinase*" (MAPK). La famille de la MAPK est

un important groupe de médiateurs qui transforme les signaux extracellulaires en réponses intracellulaires. Au moins trois différents isoformes ont été décrits : *l'extracellular signal-regulated kinase* (ERK), la kinase p38 et le "c-jun N-terminal kinase" (JNK). Edwards et coll. (2001 ; 2003) ont démontré l'implication de deux membres de la MAPK, ERK et JNK, qui sont activés suite à la phosphoryaltion par la PKC et sont impliqués dans la voie de signalisation des récepteurs I_1. D'autres études ont montré que la stimulation des récepteurs aux imidazolines par la moxonidine, dans les cellules PC12, peut activer ou non la cascade d'ERK dépendamment de la présence des autres stimuli. Cette inactivation est due à l'induction de certains phosphotasses (MPK2) pour convertir l'ERK phosphorylé de son état actif à l'état non phosphorylé (non active) (Edwards et Ernsberger, 2003). La voie de signalisation de MAPK amplifie et intégre les signaux de divers stimulis et élicite des réponses physiologiques approriées, incluant la prolifération cellulaire, la différentiation, le développement, les réponses inflammatoires et l'apoptose chez les cellules des mamifères.

D'autre part, le deuxième système de transduction est représenté par la voie de l'adénylate cyclase. Greney et coll. (2000) ont montré que la benazoline, un ligand sélectif des récepteurs aux imidazolines I_1, diminue le taux d'AMPc stimulé par la forskoline dans les cellules PC12 (où les récepteurs I_1 sont présents et non pas les récepteurs α_2-adrénergiques) mais pas dans les cellules HT 29 (cellules du cortex cérébral humain, où on trouve les récepteurs α_2-adrénergiques et pas les récepteurs I_1).

D'autres voies de signalisation peuvent exister : le groupe de Musgrave (1995; 1996) ont démontré que plusieurs composés imidazoliniques, incluant la clonidine, bloquent l'entrée des ions (Na^+ et Ca^{2+}) induite par la nicotine, cet effet est médié par la liaison des composés sur les sites de liaisons des cations dans les canaux des récepteurs acétylcholine nicotiniques (Musgrave et coll., 1996; 1995; Musgrave et Hughes, 1998). D'autres études suggèrent que les récepteurs aux

imidazolines rénaux peuvent être couplés à l'échangeur Na^+/H^+ (Bidet et coll., 1990).

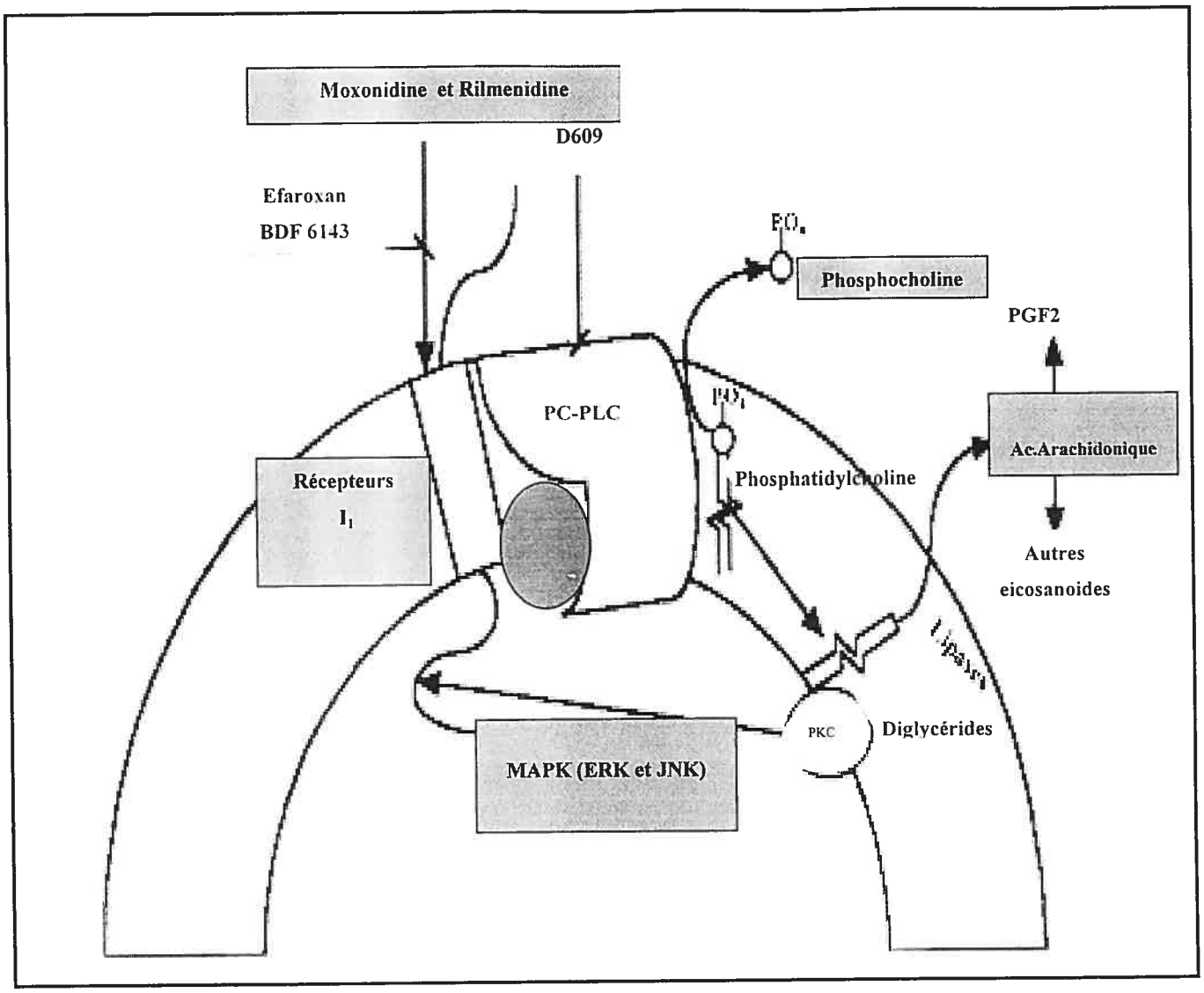


Figure 3 : la voie de signalisation des récepteurs I_1 proposée par Ernsberger, 1999.

1.2.2.6-**Fonctions des récepteurs I_1**

Le rôle physiologique des récepteurs aux imidazolines n'est pas entièrement clair. Vu que les agonistes endogènes ne sont très bien caractérisés, dans toutes les expériences, la fonction accordée aux récepteurs aux imidazolines était mise en évidence suite à l'ajout des agonistes externes.

Les récepteurs I_1 cérébraux sont directement associés à une fonction précise, la modulation de l'activité sympathique d'origine centrale, donc la régulation du tonus vasomoteur. Les récepteurs dans la région RVLM jouent un rôle important dans le mécanisme d'action hypotensive de substances telles la clonidine et la moxonidine (Bousquet et coll., 1984; Ernsberger et Haxhiu, 1997; van Zwieten, 1997).

L'activation des récepteurs aux imidazolines dans la glande surrénale, par la clonidine et ses analogues, permet la régulation de l'expression du gène de l'enzyme phényléthanolamine N-méthyltransférase (PNMT), une enzyme de biosynthèse de la norépinéphrine et de l'épinéphrine (Evinger et coll., 1995).

Le développement récent de la moxonidine (4-chloro-5-(imidazolidin-2-ylidenimino)-6-methoxy-2-methylpyrimidine), un composé imidazolinique qui se lie aux récepteurs I_1 centraux avec une affinité 30 fois supérieure à celle des récepteurs α_2-adrénergiques, offre un nouvel outil pour établir la fonctionnalité de récepteur I_1 (Ernsberger et coll., 1994).

La moxonidine est un composé antihypertensif qui réduit la pression artérielle par l'activation sélective des récepteurs aux imidazolines I_1 dans le SNC (Haxhiu et coll., 1994; Ernsberger et Haxhiu, 1997) avec une moindre contribution des récepteurs α_2-adrénergiques centraux. La moxonidine induit une inhibition du tonus sympathique chez les patients et les animaux hypertendus et chez les rats avec insuffisance cardiaque (Schachter, 1999; Mall et coll., 1991). Cette inhibition était révélée par la réduction de la fréquence cardiaque, du niveau de l'épinéphrine, de la norépinéphrine et de l'activité rénine plasmatique chez les patients hypertendus (Mitrovic et coll.,

1991; Kirch et coll., 1990). La réduction de l'activité rénine plasmatique et par conséquent la réduction de la concentration de l'angiotensine II préviennent la rétention du sodium et de l'eau, qui contribuent à la régulation aiguë et à long terme de la pression artérielle. La microinjection d'harmane, qui possède d'affinité seulement pour les récepteurs aux imidazolines, dans la RVLM chez les rats Sprague-Dawley provoque une diminution de la pression sanguine (Musgrave et Badoer, 2000). Cet effet hypotenseur de l'harmane était inhibé par l'efaroxan, un antagoniste des récepteurs aux imidazolines. Récemment, une étude par Gerova et Török (2004) a montré que l'agmatine administré intraveineux, induit une diminution de la pression artérielle et que cet effet était augmenté après l'inhibition chronique et aiguë de NO synthase.

Les récepteurs I_1 semblent aussi impliqués dans la modulation de la natriurèse, plus particulièrement dans la régulation du débit urinaire et de l'excrétion sodique (Penner et Smyth, 1994). La moxonidine exerce des actions directes sur les récepteurs I_1 rénaux pour induire la diurèse et la natriurèse (Allan et coll., 1993; Penner et Smyth, 1994; Smyth et Penner, 1995; 1999).

Les études de Smyth et Penner (1995) ont montré que la perfusion de l'agmatine, à des doses n'affectant pas la pression artérielle et la fréquence cardiaque, a provoqué une augmentation du débit urinaire et de la clairance osmolaire. Donc, cette augmentation de l'excrétion sodée observée après la perfusion d'agmatine, qui est un ligand endogène pour les récepteurs I_1 indique que ces récepteurs pourraient jouer un rôle important dans la régulation physiologique des mouvements électrolytiques rénaux.

Mukaddam-Daher et Gutkowska (1999; 2000) ont démontré que l'administration intraveineuse de la moxonidine, chez les rats normotendus et hypertendus, était associée à une diminution de la pression sanguine, à une stimulation de l'excrétion d'eau, de sodium, de potassium, de cGMP, de l'indice de l'activité du peptide natriurétique auriculaire (l'ANP) et à une augmentation du taux plasmatique de

l'ANP. Ces études **suggèrent** que l'ANP puisse être impliqué dans les effets aigus de la moxonidine. Cependant, les mécanismes impliqués dans la libération de l'ANP par la moxonidine ne sont pas connus.

Comme le cœur est le site principal de la production de l'ANP, nous avons proposé la présence de récepteurs aux imidazolines I_1 dans le cœur et que ces récepteurs cardiaques peuvent-être impliquer dans la libération des peptides natriurétiques, qui jouent un rôle intégral dans l'homéostasie de volume et de la pression dans les conditions physiologiques et pathophysiologiques.

1.3-OBJECTIFS GÉNÉRAUX DE LA THÈSE

En se basant sur les études ci-dessus, nous avons proposé que le cœur possède les récepteurs aux imidazolines et les récepteurs α_2-adrénergiques et que ces récepteurs sont fonctionnels puisque leur activation stimule la libération d'ANP. Ces études sont proposées pour démontrer qu'en plus du système nerveux central, le cœur joue un rôle dans les pathophysiologies de l'hypertension dans lesquelles les récepteurs aux imidazolines cardiaques jouent un rôle important dans la régulation de la fonction cardiovasculaire.

Les objectifs spécifiques incluent :

1) Déterminer les effets du traitement chronique à la moxonidine sur la pression artérielle, les actions rénales, le niveau plasmatique et la synthèse des peptides natriurétiques.

2) Évaluer les effets du traitement aigu à la moxonidine sur la pression artérielle, la fonction rénale et le niveau plasmatique des peptides natriurétiques chez les rats normotendus et hypertendus, et vérifier si ces effets sont altérés lors de l'hypertension.

3) Démontrer que l'ANP est directement impliqué dans les réponses rénales suite à l'administration intraveineuse de la moxonidine.

4) Localiser et caractériser les récepteurs aux imidazolines et les récepteurs α_2-adrénergiques dans le cœur. Ensuite, démontrer leur régulation lors des désordres cardiovasculaires et suite à l'exposition chronique à l'agoniste sélectif.

5) Vérifier la fonctionnalité des récepteurs I_1 et α_2-adrénergiques cardiaques et leur implication directe dans la libération de l'ANP sans l'influence du système nerveux central.

CHAPITRE 2

Le traitement chronique des SHR à la moxonidine (minipompes Alzet, sous-cutané), diminue la pression artérielle (mésurée par télémétrie), augmente la synthèse cardiaque (RT-PCR) et le niveau plasmatique des peptides antriurétiques (ANP &BNP) et résulte en une régréssion de l'hypertrophie ventriculaire gauche. Ceci nous amène à suggérer que la moxonidine peut stimuler la synthèse et la libération des peptides natriurétiques, qui à leur tour peuvent contribuer dans la régulation chronique de la pression artérielle et dans la cardioprotection.

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CHAPITRE 2

CHRONIC IMIDAZOLINE RECEPTOR ACTIVATION IN SPONTANEOUSLY HYPERTENSIVE RATS

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Menaouar et al: Chronic activation of Imidazoline Receptors in SHR
Index words: Moxonidine, ANP, BNP, blood pressure, osmotic minipumps, telemetry, LVH, natriuresis.

2.1-ABSTRACT

Acute intravenous administration of moxonidine, an imidazoline I_1-receptor agonist, reduces blood pressure in normotensive and hypertensive rats, induces diuresis and natriuresis and stimulates plasma atrial natriuretic peptide (ANP). In these studies we investigated the involvement of natriuretic peptides (ANP & BNP) in the effects of chronic activation of imidazoline receptors. Spontaneously hypertensive rats (SHR, 12-14 weeks old) received 7-day moxonidine treatment at various doses (10, 20, 60 and 120 μg/kg/h) via subcutaneously implanted osmotic minipumps. Hemodynamic parameters, continuously monitored by telemetry, revealed that compared to saline treated rats, moxonidine dose-dependently decreased blood pressures. Maximum blood pressure lowering effect was achieved by the 4^{th} day of treatment, where 60 μg/kg/h reduced MAP by 14.5±6.8 mm Hg as compared to basal levels. The decrease in MAP was influenced by a drop in both diastolic and systolic pressures. Moxonidine treatment did not alter daily urinary sodium and potassium excretions, but 120 μg/kg/h moxonidine decreased urine volume after 2 days and increased cGMP excretion on days 4 to 7 of treatment. Chronic moxonidine treatment dose-dependently increased plasma ANP to reach at 120 μg/kg/h 40% increase ($P<0.01$) above corresponding saline-treated SHR, with a concomitant increase in left and right atrial ANP mRNA (>2 fold). Plasma BNP increased by 120 μg/kg/h moxonidine (11.0±1.1 vs. 16.5±1.9 pg/ml, $p<0.002$) without significant increases in atrial and ventricular BNP mRNA. In conclusion, ANP and BNP may be involved in the antihypertensive effect of chronic moxonidine treatment. Accordingly, natriuretic peptides may contribute to the sympatholytic and cardioprotective effects of chronic activation of imidazoline I_1-receptors.

2.2-INTRODUCTION

Activation of the sympathetic outflow to the heart, kidneys and skeletal muscle vasculature is commonly present in young (<45 years) patients with essential hypertension. The sympathetic stimulation leads to cardiac risks, such as development of left ventricular hypertrophy (LVH), predisposing to ventricular arrythmias, increasing insulin resistance and accelerating atherogenesis. Consequences of increased sympathetic activity and elevated levels of catecholamines in essential hypertension can be minimized by reduction of sympathetic outflow by centrally acting antihypertensive agents. However, despite their efficacy, the use of these drugs has been often limited by their adverse effects, such as dry mouth and sedation. Recently, newly developed centrally acting antihypertensive drugs with less adverse effects, i.e. moxonidine and rilmenidine, which show high affinity for the non-adrenergic imidazoline I_1-receptors and low affinity for α2-adrenergic receptors, have been introduced in hypertension treatment. These drugs reduce blood pressure by selective activation of I_1-receptors in the central nervous system,[1,2] inducing an inhibition of sympathetic tone,[3,4] and by direct actions on imidazoline receptors in the kidney to cause diuresis and natriuresis,[5,6] both mechanisms leading to acute and long-term control of pressure.

Studies from this laboratory have shown that acute intravenous moxonidine administration to normotensive[7] and spontaneously hypertensive rats[8] reduces blood pressure, increases plasma atrial natriuretic peptide (ANP) and stimulates urine flow and the excretion of sodium, potassium and cGMP, the index of ANP activity, indicating that ANP is involved in the acute effects of moxonidine.

The natriuretic peptides, ANP and BNP, primarily produced by the cardiac atria and ventricles, have potent diuretic, natriuretic and vasorelaxant activities. Natriuretic peptides inhibit cell proliferation and extracellular matrix production of cardiac fibroblasts through inhibition of the renin-angiotensin and endothelin systems.[9]

The actions of these peptides are mediated by a common guanylyl cyclase coupled natriuretic peptide receptor (NPR-A) leading to increased intracellular cGMP production, and another clearance (NPR-C) receptor through inhibition of adenylyl cyclase/cAMP.

Due to their established role in volume and pressure regulation, natriuretic peptides may contribute to the antihypertensive effects of chronic activation of imidazoline receptors by moxonidine. Therefore, the cardiovascular and renal effects of chronic moxonidine therapy and the possible involvement of natriuretic peptides in these effects were investigated in conscious spontaneously hypertensive rats (SHR), a model of human essential hypertension.

2.3-METHODS

Female Spontaneously Hypertensive rats (SHR, 12-14 weeks old) and normotensive Sprague-Dawley rats (SD, 200-225 g) were purchased from Charles River (St. Constant, Quebec). The animals were housed at 22°C, maintained on a 12-h light/12-h dark cycle, and fed Purina Rat Chow (Ralston Purina) and tap water *ad libitum* for at least 3 days before experimentation. All procedures were carried out with the approval of the Bioethics Committee of CHUM, according to the Canadian Guidelines.

Hemodynamic Measurements

Blood pressures, systolic, diastolic and mean arterial pressure (MAP), HR, and locomotor activity in freely moving rats were measured by telemetry (Data Sciences International, St. Paul, MN) as we have previously described.[7,10] The animals were anaesthetized with pentobarbital, and the flexible transmitter catheter was secured surgically in the abdominal aorta below the renal arteries pointing against the flow. The transmitter was sutured to the abdominal wall. The rats were placed separately in cages and each cage was placed over the receiver panel and connected to the computer for collection of data. The monitoring system consisted of a pressure transmitter (radio frequency transducer model TL11M2-C50-PXT), receiver panel, consolidation matrix, and personal computer with program. Output from the transmitter was monitored by the receiver (RLA 2000). The signals from the receiver were consolidated by the multiplexer (BCM 100) and were stored and analyzed by microcomputer with software (A.R.T., Dataquest). The pressure signals were corrected automatically for changes in atmospheric pressure.

The animals were allowed to recover for at least 10 days before experimentation. Then, under isoflurane anesthesia, animals were randomly implanted subcutaneously with osmotic minipumps (2ML1, Alzet Corp., USA) that allowed continuous delivery of moxonidine (10, 20 and 60 μg/kg/h) or vehicle at the rate of 10 μL/h, for 1 week.

Rats were placed in their cages over corresponding pressure receivers. Data from all parameters were collected every min over 24 h before and for 7 days during moxonidine or vehicle treatment.

Moxonidine (generous gift of Solvay Pharmaceuticals, GMBH) solution was prepared by dissolving the drug in isotonic saline, acidified with 1 N HCL (pH < 6.5), then adjusted to pH 7.0-7.4 with 1 N NaOH.

Renal effects of moxonidine

In other groups of normotensive and hypertensive rats, the renal responses to various moxonidine treatments were investigated by assessment of diuresis, natriuresis, and kaliuresis as well as urinary cGMP (UcGMP) excretion. Animals were placed in metabolic cages for 3 days before experimentation. Then, they were randomly separated into 4 groups and implanted with osmotic minipumps (as described above), containing either moxonidine (10, 60, or 120 μg/kg/h) or saline vehicle. The rats were re-placed in their metabolic cages. Body weight, water and food intake and 24 h urine output were measured daily at 8:30 a.m., 2 days before- and over 7 days of moxonidine or vehicle treatment.

Sodium and potassium concentrations were measured in urine with a flame photometer (Instrumentation Laboratory, Lexington, MA). Urinary cGMP was measured in serial dilutions by a specific RIA as previously described.[10]

Plasma and Tissue Natriuretic Peptide Measurement

The animals were sacrificed by decapitation on day 7 of moxonidine treatment. Trunk blood (1 ml) was collected in pre-chilled tubes containing protease inhibitors in a final concentration: 10 μM EDTA, 10 μM phenylmethylsulfonyl fluoride (PMSF) and 5 μM pepstatin A (Sigma Chemical Co. St. Louis, MO, USA). After blood centrifugation at 4°C, plasma was collected and stored at -80°C. The hearts were rapidly excised and atria and ventricles were dissected and frozen in liquid nitrogen then stored at -80°C.

For measurement of natriuretic peptide content, the tissues were thawed in 0.1 M acetic acid containing protease inhibitors (as above) at 4°C. The tissues were homogenized twice in a Polytron homogenizer, and centrifuged at 30,000 g for 20 min at 4°C. The retrieved supernatants were combined and stored at -80°C.

ANP and BNP were measured by radioimmunoassay[11,12] in plasma after extraction by Sep-Pak C18 cartridges (Millipore, Mississauga, Ont. Canada) and in serial dilutions of

tissue homogenates using specific antibodies. Rat ANP_{1-28} and BNP-32 were iodinated with ^{125}I-Na using lactoperoxydase and purified by high performance liquid chromatography. Proteins were measured spectrophotometrically (absorbance 595 nM), using bovine serum albumin (BSA) as standard.

Total RNA Extraction and RT-PCR.

ANP and BNP gene expression in the heart chambers were detected by RT-PCR as we previously described.[12] Total RNA was isolated from the rat hearts using TRIZOL reagent (Life Technologies, Inc.) according to the manufacturer's specifications and treated with RNase-free DNase I under a standard protocol. The integrity and quality of the purified RNA were controlled by formaldehyde denaturing agarose gel electrophoresis and by measurement of the A260/A280 nm ratio. First strand cDNA was synthesized in a final volume of 40 µl containing first strand buffer, 2 µg rat cardiac RNA used as a control, 2 µg hexanucleotide primer (Pharmacia), and avian myeloblastosis virus (AMV) reverse transcriptase (12 units/µg RNA; Life Technologies, Inc.).

For PCR amplification the forward ANP primer spanned the junction of exon 1 and 2 of ANP gene (bp170-198) and reverse (bp 527-494) spanned the junction of exon 2 and 3 of ANP DNA. The forward BNP primer (bp -36 to -4) spanned sequences in the 5'-untranslated region on the first exon and reverse primer spanned translated (bp 334-316) sequence on the second exon of rat BNP gene. Ten µl of the PCR products were electrophoresed on 1.5 agarose gel in the presence of ethidium bromide. Fluorescent bands were counted and analyzed with the Storm 840 Imaging System and ImageQuant software (Version 5.1, Molecular Dynamics). To validate the use of this RT-PCR assay as a tool for the semiquantitative measurement of ANP mRNA and BNP mRNA, dose-response curves were established for different amounts of total RNA, and the samples were quantified in the curvilinear phase of PCR amplification. These data were normalized to the corresponding values of 18S RNA PCR products in the same samples (amplified by RT-PCR with primers manufactured by Ambion Inc.).

Statistical analysis

Telemetric data obtained each 1-min from each rat over 24 h were pooled. All parameter values were expressed as difference from baseline. Comparisons between groups were made by analysis of variance (ANOVA). Paired Student's t-test was used in each group to compare initial to final values, and non-paired t-test to compare values in moxonidine treated rats vs. corresponding saline controls. $P<0.05$ was considered significant.

All data are expressed as means ± SEM.

2.4-RESULTS

Compared to saline treated rats, one-week treatment with moxonidine had no significant effect on water and food intake, neither on body weight. However, to rule out any effect of body weight difference among the various groups, all renal parameters were normalized to percent body weight.

Compared to basal, urine volume decreased on the first day of saline or 10 and 60 μg/kg/h moxonidine treatment, but attained balance by 4 days. The dose of 120 μg/kg/h reduced urine excretion from a basal of 14.3 ± 0.6 to 11.2 ± 0.9 ml ($p < 0.05$) on day 7. On the other hand, cGMP increased (160%, $p<0.04$) by 120 μg/kg/h moxonidine starting on day 4 but not significantly changed thereafter. Urinary sodium and potassium concentrations significantly increased by treatment as compared to basal values. However, the increase disappeared when daily excretions were calculated. Serum creatinine levels were not altered by either treatment, indicating maintained renal function.

During the first 2-days of treatment, all groups, including saline-treated controls, showed a transient decrease from basal values in activity pattern, but returned to normal for the remaining period of treatment.

Basal pressure measurements were not different among the various hypertensive groups. Basal systolic pressure in all SHR was 170.3±4.2 mm Hg, diastolic pressure, 118.4±3.1 mm Hg, and MAP, 143.4±3.6 mm Hg. Figure 1 shows that compared to basal values, treatment with 10, 20 and 60 μg/kg/h moxonidine resulted in a dose-dependent reduction in systolic, diastolic and mean arterial pressures. Maximum blood pressure lowering effect was achieved by the 4^{th} day of treatment, but thereafter, treatment did not cause any further reduction in pressures. Moxonidine at 60 μg/kg/h reduced MAP on day 4 by 14.5±6.8 mm Hg from basal. The decrease in MAP was influenced by a drop in both diastolic and systolic pressures (Figure 1).

Baseline HR in SHR was 349±7 beats/min. Figure 1 shows that there were no significant bradycardic effects of various doses of moxonidine in spontaneously hypertensive rats.

The hypertensive rats showed a higher left ventricular weight to body weight (LV/BW) ratio than normotensive rats (2.32±0.08 vs. 3.06±0.05 mg/g, $p<0.001$), indicating left

ventricular hypertrophy in 12-14 week old SHR. LV/BW ratio was not altered by 7-day treatment with 120 μg/kg/h of moxonidine in normotensive and hypertensive rats. However, treatment resulted in a significant decrease in left ventricular weight in hypertensive rats (585.30 ± 0.01 vs 542.40 ± 0.01 mg, $p<0.004$).

Basal plasma ANP levels were significantly higher in SHR than normotensive rats (104±10 vs. 398±32 pg/ml, $p<0.001$). Chronic treatment of SHR with moxonidine (10, 60 and 120 μg/kg/h) induced further dose-dependent increases in plasma ANP and represented with the highest dose of 120 μg/kg/h a 40% increase ($P<0.01$) above corresponding saline-treated hypertensive controls (Figure 2).

Plasma BNP was also significantly higher in SHR than normotensive rats (6.2±0.4 vs. 11.0±1.1 pg/ml, $p<0.001$). Moxonidine dose-dependently stimulated plasma BNP, so that it represented 16.5±1.9 pg/ml ($p<0.002$) at 120 μg/kg/h (Figure 2).

Tissue ANP and BNP content was measured in atria and ventricles from normotensive SD rats and hypertensive SHR treated with saline-vehicle or 120 μg/kg/h moxonidine for 7 days. ANP was lower in both ventricles and in left, but not right, atria of SHR as compared to normotensive rats. However, after chronic treatment of SHR with moxonidine, ANP concentrations decreased significantly in left ($P<0.01$) and right ($P<0.001$) atria (Figure 3), and remained unchanged in ventricles.

Hypertension decreased BNP content in left but not right atria. Treatment with moxonidine resulted in a further decrease in left atrial BNP content (6.0±0.5 vs. 2.7±0.2 ng/mg protein, $p<0.03$), without affecting right atrial or ventricular levels.

ANP and BNP mRNA were measured in atria and ventricles from SHR treated with moxonidine (10, 60 and 120 μg/kg/h) for 7 days. Moxonidine treatment induced significant and dose-dependent increases in left and right atrial ANP mRNA when compared to saline-treated rats. Figure 4 shows that 120 μg/kg/h moxonidine increased ANP mRNA by 2.1 and 2.4 fold in right and left atria, respectively. Moxonidine also increased ANP mRNA by 26% in left ventricles (data not shown). On the other hand, moxonidine did not change BNP mRNA in SHR left and right atria, but resulted in a 24% increase in left ventricles.

3.5-DISCUSSION

The present study shows that chronic treatment of hypertensive rats with moxonidine decreases blood pressure at doses that have minimal effects on animal activity, indicating reduced or absence of sedation, normally observed with α2-adrenergic agonists.[13-15] The decrease in pressure is associated with increased excretion of urinary cGMP, and increased atrial synthesis and release of natriuretic peptides, ANP and BNP. These findings support the notion that, in addition to activation of imidazoline receptors in the brain and kidneys, the chronic antihypertensive action of moxonidine may include stimulation of natriuretic peptides, which would contribute to the maintenance of blood pressure reduction and cardioprotection by imidazoline compounds.

Selective activation of central imidazoline I_1-receptors, located in the rostral ventro-lateral medulla (RVLM) results in inhibition of peripheral sympathetic activity and produces arterial vasodilation.[1-5] In the present study, chronic activation of imidazoline I_1-receptors by moxonidine at various doses attenuated the high blood pressure, starting by the first day of treatment, and lower blood pressure was maintained throughout the 7 days of the study. The magnitude of reduction in systolic and diastolic blood pressures was within the range previously reported by others in SHR treated orally with moxonidine at 8 to 10 mg/kg/day.[16,17]

Stimulated natriuretic peptides would contribute to the pressure lowering effect of moxonidine through inhibiting sympathetic activity and decreasing total peripheral resistance.[18,19] The sympatholytic effect of ANP has been shown in transgenic mice where a 20-fold increase in plasma ANP was associated with a reduction of 20 mm Hg in blood pressure,[20] whereas mice with a homozygous disruption of the Pro-ANP gene (-/-) that fail to synthesize ANP developed chronic hypertension due to elevated total peripheral resistance as determined by an increase in cardiovascular autonomic tone.[21]

Similarly, transgenic mice over-expressing BNP exhibit reduced blood pressure that is accompanied by an elevation of plasma cGMP concentrations, and BNP-transgenic mice lacking natriuretic peptide receptor A (NPR-A) are hypertensive.[22] Therefore, it is reasonable to suggest that moxonidine–stimulated ANP and BNP may contribute to attenuation of sympathetic tone by moxonidine.

The blood pressure-lowering effect of centrally acting imidazoline receptor agonists is associated with inhibition of renal sympathetic nerve activity, and subsequent diuresis and natriuresis.[6] These enhanced renal responses have been observed after acute[7,8] but not chronic moxonidine treatment.[23] It is important to note that imidazoline antihypertensive agents are not diuretics. However, unlike other sympatholytic agents, imidazoline compounds do not cause sodium retention,[24] suggesting the presence of intrarenal mechanisms counteracting sodium-retaining effects that result from sympatho-inhibition and lower blood pressure. In support, a shift in the pressure natriuresis curve was observed in rats after rilmenidine (selective I_1-receptor agonist) treatment, indicating a significant contribution of renal mechanisms to long-term blood pressure control by I_1-receptor agonists.[25] Renal mechanisms that could contribute to the maintenance of sodium balance may include direct renal action of the drug. In fact, I_1-imidazoline receptors have been found in the kidney,[1] and direct infusion of moxonidine into the renal artery increases sodium and water excretion.[6,26] In addition, treatment-stimulated natriuretic peptides would act on their own receptors in the kidney to maintain sodium balance.

Previous studies have shown that SHR develop LVH at 4 weeks of age, even before blood pressure starts to increase. In the present study, 12-14 week old SHR with established hypertension showed elevated left ventricular to body weight ratio, confirming development of left ventricular hypertrophy. Although relatively short, the 7 day treatment decreased left ventricular weight, implying that treatment of a longer duration may have further beneficial effects. The reversal of left ventricular hypertrophy may be influenced by stimulated natriuretic peptides.

Independent of their role in blood pressure control, natriuretic peptides have direct antihypertrophic and antifibrotic actions on the heart.[27] The NPR-A system has intrinsic growth inhibitory properties in non-cardiac and cardiac cells *in vitro,*[28] and in endothelial and vascular smooth muscle cells, ANP is antimitogenic.[29] Mice lacking NPR-A display a marked cardiac hypertrophy and chamber dilatation by 3 months of age,[30] and mice with disrupted BNP develop multifocal fibrotic lesions in the ventricles.[31]

Taken together, these data show that chronic activation of imidazoline receptors in hypertensive rats is associated with stimulated production and synthesis of natriuretic peptides, which may contribute to the long-term sympatholytic and cardioprotective effects of the treatment.

Acknowledgements

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2.7-FIGURE LEGENDS

Figure 1: Effect of 7-day treatment of SHR with different doses of moxonidine (10, 20 and 60 μg/kg/h) on hemodynamic parameters (n = 2 to 6 rats/group), represented as difference from basal. MAP: mean arterial pressure, HR: heart rate, Moxo: moxonidine.

Figure 2: Dose-dependent effect of 7-day moxonidine treatment (10, 60 and 120 μg/kg/h) on plasma ANP and BNP in SHR. *$p<0.02$ vs. Sprague-Dawley (SD) normotensive control rats receiving saline vehicle; **$p<0.02$ vs. saline-treated SHR; n = 7- 20 rats/group.

Figure 3: Effect of 7-day moxonidine treatment (120 μg/kg/h) on left and right atrial ANP content in SHR. *$p<0.01$, **$p<0.001$ vs. saline-treated SHR; n = 4 rats/group.

Figure 4: Bargraphs represent dose-dependent effect of chronic moxonidine treatment (10, 60 and 120 μg/kg/h) on left and right atrial ANP mRNA in SHR as normalized to 18S mRNA. *$p<0.001$ vs. saline-treated SHR; n = 6 rats/group.

Figure 1

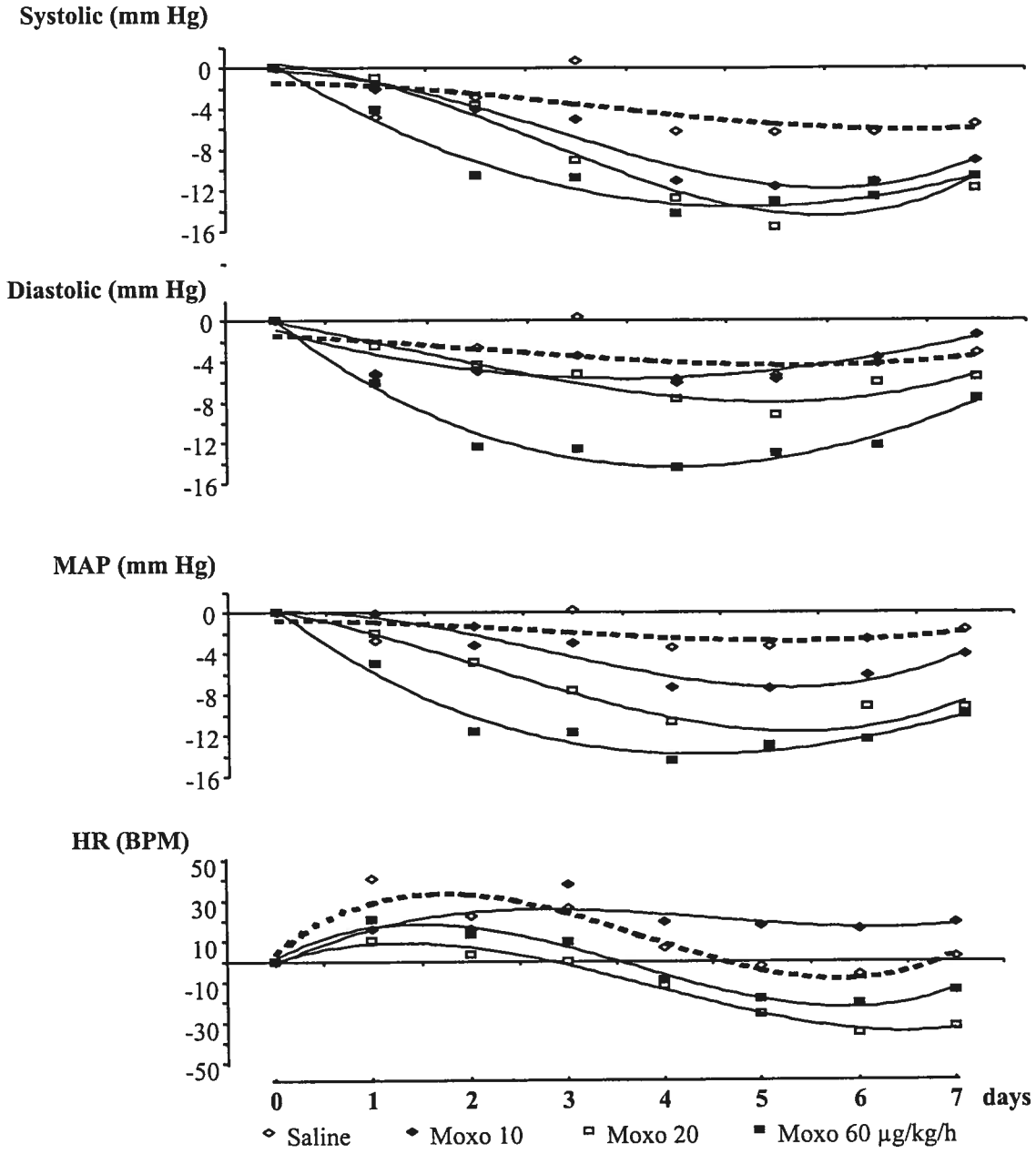

Systolic (mm Hg)
0
-4
-8
-12
-16
Diastolic (mm Hg)
0
-4
-8
-12
-16
MAP (mm Hg)
0
-4
-8
-12
-16
HR (BPM)
50
30
10
-10
-30
-50
0 1 2 3 4 5 6 7 days
Saline
Moxo 10
Moxo 20
Moxo 60 µg/kg/h

Figure 2

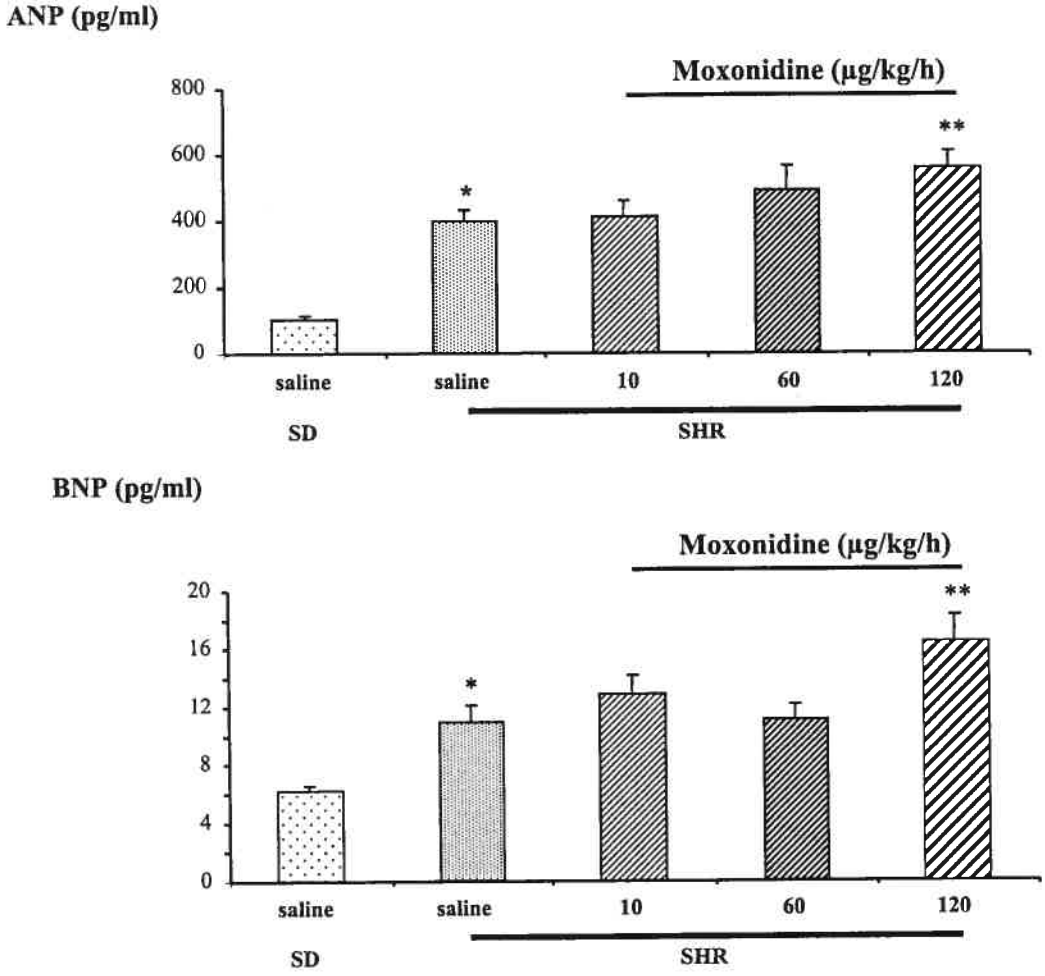

ANP (pg/ml)
Moxonidine (µg/kg/h)
800
600
400
200
0
*
**
saline
saline
10
60
120
SD
SHR
BNP (pg/ml)
Moxonidine (µg/kg/h)
20
16
12
8
4
0
*
**
saline
saline
10
60
120
SD
SHR

Figure 3

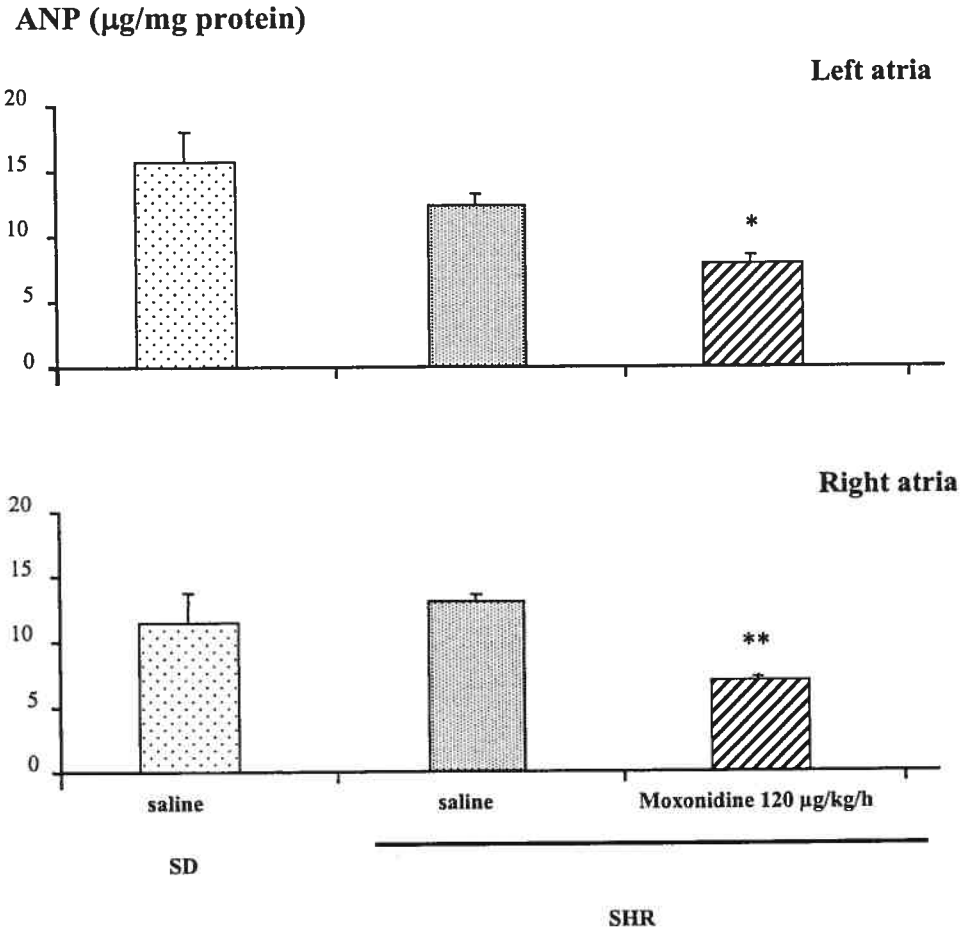
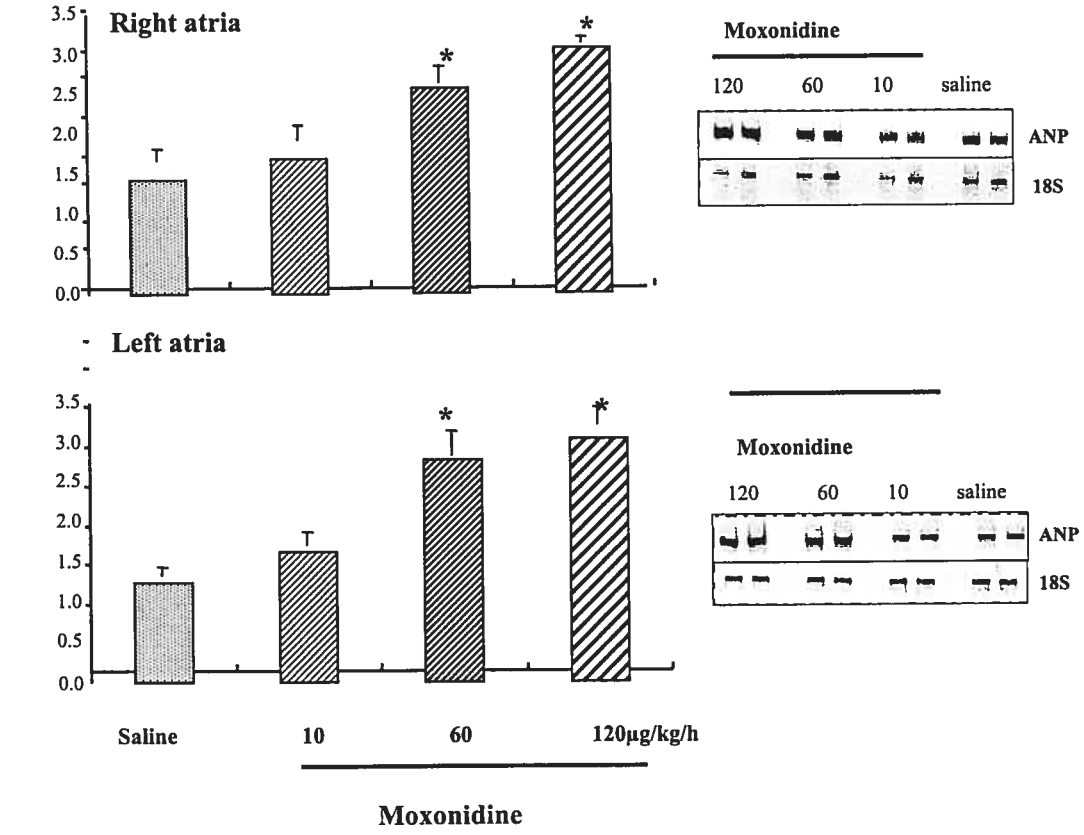

ANP (µg/mg protein)
Left atria
20
15
10
5
0
*
Right atria
20
15
10
5
0
**
saline
saline
Moxonidine 120 µg/kg/h
SD
SHR

Figure 4


ANP mRNA
Right atria
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
Moxonidine
120
60
10
saline
ANP
18S
Left atria
Moxonidine
120
60
10
saline
ANP
18S
Saline
10
60
120μg/kg/h
Moxonidine

CHAPITRE 3

Lors du traitement aigu, l'injection intraveineuse de la moxonidine chez les rats normotendus et hypertendus conscients stimule la diurèse, la natriurèse, l'excrétion de GMPc, un marqueur de l'activité d'ANP, ceci suggère l'implication de l'ANP, une hormone diurétique et natriurétique dans les réponses rénales. Les effets rénaux provoqués par la moxonidine étaient inhibés de façon dose-dépendante par l'anantin, un antagoniste des peptides natriurétiques. En effet, cette étude a signalé pour la première fois l'implication directe de de l'ANP dans les actions rénales aiguës de la moxonidine.

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CHAPITRE 3

URINARY RESPONSES TO ACUTE MOXONIDINE ARE INHIBITED BY NATRIURETIC PEPTIDE RECEPTOR ANTAGONIST

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El-Ayoubi et al: ANP and renal effects of moxonidine

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Index words: Moxonidine, Clonidine, Natriuretic peptides, Imidazoline receptors, α_2-adrenoceptors, Natriuresis, cGMP, SHR, Anantin.

Abbreviations
Imidazoline I1 receptors (I_1-receptors), Atrial natriuretic peptide (ANP), Brain natriuretic peptide (BNP),Natriuretic peptide receptors (NPR), Spontaneously hypertensive rats (SHR), Wistar-Kyoto (WKY),Sprague-Dawley (SD)

3.1-ABSTRACT

1 We have previously shown that acute intravenous injections of moxonidine and clonidine increase plasma atrial natriuretic peptide (ANP), a vasodilator, diuretic and natriuretic hormone. We hypothesized that moxonidine stimulates the release of ANP, which would act on its renal receptors to cause diuresis and natriuresis, and these effects may be altered in hypertension.

2 Moxonidine (0, 10, 50, 100 or 150 μg in 300 μl saline) and clonidine (0, 1, 5 or 10 μg in 300 μl saline) injected intravenously in conscious normally-hydrated normotensive Sprague-Dawley rats (SD, ~200 g) and 12-14-week-old Wistar-Kyoto (WKY) and spontaneously hypertensive rats (SHR), dose-dependently stimulated diuresis, natriuresis, kaliuresis and cGMP excretion, with these effects being more pronounced during the 1st h post-injection. The actions of 5 μg clonidine and 50 μg moxonidine were inhibited by yohimbine, an α_2-adrenoceptor antagonist, and efaroxan, an imidazoline I_1-receptor antagonist.

3 Moxonidine (100 μg) stimulated ($P<0.01$) diuresis in SHR (0.21±0.04 vs 1.16±0.06 ml h^{-1} 100 g^{-1}), SD (0.42±0.06 vs 1.56±0.19 ml h^{-1} 100 g^{-1}) and WKY (0.12±0.04 vs. 1.44±0.21 ml h^{-1} 100 g^{-1}). Moxonidine-stimulated urine output was lower in SHR than in SD and WKY. Moxonidine-stimulated sodium and potassium excretions were lower in SHR than in SD, but not WKY, demonstrating an influence of strain but not of pressure. Pretreatment with the natriuretic peptide antagonist, anantin (5 or 10 μg) resulted in dose-dependent inhibition of moxonidine-stimulated urinary actions. Anantin (10 μg) inhibited ($P<0.01$) urine output to 0.38±0.06, 0.12±0.01, and 0.16±0.04 ml h^{-1} 100 g^{-1}in SD, WKY and SHR, respectively. Moxonidine increased ($P<0.01$) plasma ANP in SD (417±58 vs 1,021±112 pg ml^{-1}) and WKY (309±59 vs 1,433±187 pg ml^{-1}), and in SHR (853±96 vs 1,879±229 pg ml^{-1}).

4 These results demonstrate that natriuretic peptides mediate the urinary actions of moxonidine through natriuretic peptide receptors.

3.2-INTRODUCTION

Centrally-acting antihypertensive compounds, such as clonidine, moxonidine, and rilmenidine, reduce blood pressure by acting, albeit with different affinities, on α_2-adrenoceptors and imidazoline I_1-receptors, resulting in sympathoinhibition. In addition, these drugs may directly act on the kidneys to stimulate diuresis and natriuresis, thus contributing to short- and long-term control of blood pressure (Ziegler et al., 1996; Ernsberger, 2000).

Several groups have investigated the mechanisms involved in the renal responses to acute injections of these centrally-acting compounds. Smyth and Penner (1998) have shown that intracerebroventricular administration of moxonidine produces significant increases in urine and sodium excretion that are totally blocked by intravenous prazosin, implicating inhibition of renal nerve activity and subsequent α_1-adrenoceptor stimulation in these effects. Further studies have shown that independent of the renal nerves and vasopressin, moxonidine may exert direct effects on the kidney to cause diuresis and natriuresis, (Allan et al., 1993; Smyth & Penner, 1998), by acting on its receptors in the proximal tubules (Bidet et al., 1990; Limon et al., 1992; Li & Smyth, 1993a; Bohmann et al., 1994; Greven et al, 2001) to inhibit Na^+-H^+ exchanger (Schlatter et al., 1997).

Comparing two imidazoline compounds with different affinities for I_1-receptors versus α_2-adrenoceptors, Hohage et al. (1997b) reported that intravenous moxonidine, which binds to I_1-receptors with greater affinity than clonidine, transiently increased fractional fluid and sodium excretion in anesthetized rats, whereas equal concentrations of clonidine resulted in a sustained increase in fractional fluid excretion. The effects were inhibited by selective antagonists, leading to the conclusion that the drugs acted on two different receptors (Hohage et al., 1997b).

In contrast, Hohage et al. (1997a) demonstrated, in SHR, that moxonidine, but not equal concentrations of clonidine, stimulated diuresis and natriuresis, and that the effects were long-lasting, suggesting altered response in hypertension. However, the influence of hypertension was not investigated, as the study did not include normotensive controls.

Li et al. (1994) reported that the renal effects of intravenous moxonidine were attenuated in 1 kidney-1 clip (1K1C) hypertensive rats compared to sham controls, and attributed the attenuation to down-regulation of renal imidazoline receptors in this model (Li & Smyth, 1993a).

In earlier studies, we have demonstrated that acute intravenous injections of various doses of clonidine and moxonidine in normotensive Sprague-Dawley rats evoked dose-dependent diuresis and natriuresis, and contrary to the finding of Hohage et al. (1997), clonidine was at least ten times more potent that moxonidine. The renal effects were inhibited by yohimbine as well as by efaroxan, a selective imidazoline I_1-receptor antagonist (Mukaddam-Daher & Gutkowska, 2000). We also reported preliminary results (Mukaddam-Daher & Gutkowska, 1999) from spontaneously hypertensive rats (SHR) and normotensive Wistar-Kyoto (WKY) rats, showing that moxonidine significantly stimulated diuresis and natriuresis and that the effects were totally inhibited by efaroxan and partially by yohimbine, indicating that the effects of moxonidine were primarily mediated by imidazoline receptors. Other experiments revealed that the efaroxan dose used in that study (500 μg/rat) was too high to correctly draw a conclusion on the receptor type mediating these effects. Our investigations, however, revealed that acute treatment with clonidine and moxonidine was associated with a dose-dependent increase of plasma atrial natriuretic peptide (ANP) and urinary excretion of cGMP, an index of natriuretic peptide activity. ANP is a potent vasodilator, diuretic and natriuretic hormone, primarily of cardiac origin. Accordingly, we proposed a new mechanism of action of moxonidine, namely, the involvement of natriuretic peptides in these effects. Direct proof of this hypothesis remained to be performed.

Because of controversial reports on the mechanisms and receptor type (s) involved in the renal effects of imidazoline compounds, and regulation in hypertension, the present study was designed to examine renal responses to acute intravenous moxonidine and clonidine in conscious hypertensive rats (SHR) compared to two normotensive models, Sprague-Dawley (SD) and WKY rats. The direct involvement of natriuretic peptides in these effects was demonstrated by using anantin, the first microbially-produced competitive peptide antagonist of natriuretic peptides. At doses that do not evoke agonistic effects,

anantin dose-dependently inhibits ANP-induced intracellular cGMP accumulation in bovine aorta smooth muscle cells (Weber et al., 1991; Wyss et al., 1991).

3.3-METHODS

Female SHR (12-14 weeks old) and age-matched normotensive WKY as well as normotensive SD rats (~200 g) purchased from Charles River (St-Constant, Quebec, Canada), were housed in a temperature- and light-controlled room with free access to food and water. The two normotensive control groups were used to investigate the selective effect of blood pressure without the confounding influence of genetic background. Experiments were approved by the Animal Care Committee of the CHUM, according to the Canadian Council on Animal Care guidelines.

All experiments were started in the morning (around 8:00 a.m.). One dose of moxonidine (0, 1, 10, 50, 100 or 150 μg) or the reference drug clonidine (0, 1, 5, or 10 μg) was injected into the tail vein in different groups. The injection procedure took about 60 seconds. Then, the rats were placed individually in Nalgene plastic metabolic cages (Braintree Scientific, Inc., Braintree, MA, USA) without food and water. Spontaneously-voided urine was collected every hour, over 4 consecutive hours, for the measurement of urine volume and electrolyte excretion. In other experiments, rats were injected with 50 μg moxonidine or 5 μg clonidine after 10-min pretreatment with efaroxan (250 or 25 μg) or yohimbine (50 or 25 μg) in 300 μl saline.

The contribution of natriuretic peptides to the urinary effects of 100 μg moxonidine was investigated in separate groups of rats, after 10-min pretreatment with anantin (5 or 10 μg/rat). The anantin doses were chosen in preliminary experiments on normotensive SD rats and shown to dose-dependently inhibit diuresis and natriuresis, as well as cGMP excretion, evoked by acute volume expansion (by rapid injection of 6 ml isotonic saline), the primary stimulus of natriuretic peptide release.

Separate groups of rats were sacrificed by decapitation 15-20 min after injection of moxonidine (100 μg) or an equal volume of saline vehicle.

Blood was collected in prechilled tubes containing protease inhibitors in a final concentration: 1 mmol l^{-1} EDTA, 5 μmol l^{-1} Pepstatin A and 10 μmol l^{-1} phenylmethylsulfonyl fluoride (PMSF) and immunoreactive ANP was measured in extracted plasma by a specific radioimmunoassay as described previously (Gutkowska,

1987). Urinary cGMP was quantified by radioimmunoassay according to a previously described method (Gutkowska et al., 1997). Urinary sodium and potassium concentrations were measured with a flame photometer (Perkin-Elmer 51, Norwalk, CT, USA), and excretions per hour were calculated. Although the rats had almost similar body weight (~200 g), their renal parameters were normalized to percent body weight to avoid the effect of any body weight variation among the different groups.

Drugs

Moxonidine (kindly provided by Solvay Pharmaceuticals, Hannover, Germany) was dissolved in 0.1 mol l^{-1} acetic acid, tand its volume was brought up to the required concentration with normal saline. Clonidine, efaroxan, and yohimbine (Sigma-Aldrich, St. Louis, MO, USA) were dissolved in saline. Anantin (Cedarlane Laboratories Ltd., Hornby, ON, Canada) was dissolved in 50% acetic acid, aliquoted and stored at -20°C. On the day of the experiment, aliquots were diluted to 5 or 10 μg in 300 μl saline. All solutions were freshly prepared on the day of the experiments.

Data Analysis

Statistical analysis of data obtained from normotensive SD and WKY rats and hypertensive SHR with and without different treatments was performed by ANOVA, followed by Neuman-Keuls multiple comparison test. $P<0.05$ was considered significant. All data are expressed as mean ± s.e.m.

3.4-RESULTS

Renal parameters measured over four hours post-moxonidine and clonidine in normotensive SD and WKY rats and hypertensive SHR revealed that the effects were more pronounced during the 1st hour of treatment. First hour urine output after injection of saline vehicle was higher in SD (0.42±0.06 ml^{-1} h^{-1} 100 g^{-1} body weight, n=13, P<0.001) than in WKY (0.12±0.04 ml^{-1} h^{-1} 100 g^{-1} , n=26) and SHR (0.21±0.04 ml^{-1} h^{-1} 100 g^{-1}, n=16). Increasing doses of clonidine (Figure 1) and moxonidine (Figure 2) stimulated the excretion of urine, sodium, potassium and cGMP in a dose-dependent manner, with similar profiles.

The renal responses to 5 μg clonidine and 50 μg moxonidine and inhibition by yohimbine and efaroxan are depicted in Figures 3 & 4. Yohimbine at 25 μg (data not shown) tended to but did not significantly suppress the renal parameters evoked by clonidine and moxonidine. At 50 μg, the inhibitory effect of yohimbine was more evident. Efaroxan at 25 μg significantly inhibited and at 250 μg (data not shown) it totally suppressed both clonidine- and moxonidine-stimulated renal parameters. Thus, α_2-adrenoceptors and imidazoline I_1-receptors are implicated in these renal responses.

Compared to saline vehicle, moxonidine at 100 μg significantly (p<0.001) increased the renal parameters measured over 1 hour post-injection in all groups. Moxonidine-stimulated urine output in SHR (1.16±0.06 ml^{-1} h^{-1} 100 g^{-1}, n=16) was significantly (p<0.05) lower than in SD (1.56±0.19 ml^{-1} h^{-1} 100 g^{-1}, n=10) and WKY rats (1.44±0.21 ml^{-1} h^{-1} 100 g^{-1}, n=12) (Figure 5). Sodium and potassium excretions were also significantly (P<0.05) lower in SHR compared to SD but not WKY rats.

Figure 5 also shows that pre-treatment with the natriuretic peptide receptor antagonist, anantin, dose-dependently inhibited the 1st hour renal parameters stimulated by moxonidine. At 10 μg, anantin inhibited (P<0.001) moxonidine-stimulated urine output to 0.38±0.06 ml^{-1} h^{-1} 100 g^{-1} in SD rats and to 0.12±0.01 and 0.16±0.04 ml^{-1} h^{-1} 100 g^{-1} in WKY and SHR, respectively. Similarly, anantin totally abolished moxonidine-stimulated sodium, potassium, and cGMP excretions (Figure 5).

Plasma ANP levels measured 15-20 min after moxonidine or saline-vehicle injections are shown in Figure 6. Plasma ANP tended to be higher in SHR (853±96 pg ml^{-1}, n=14) than in WKY (309±59 pg ml^{-1}, n=5) and SD (417±58 pg ml^{-1}, n=18) rats. Moxonidine stimulated plasma ANP in SHR (1,878±229 pg ml^{-1}, n=14, $P<0.001$) and WKY (1,433±187 pg ml^{-1}, n=5, $P<0.01$) to higher levels than corresponding SD (1,021±112 pg ml^{-1}, n=19).

3.5-DISCUSSION

The results of the present study indicate, in conscious freely-voiding rats, that: 1) acute intravenous administration of moxonidine and clonidine evokes diuresis, natriuresis, kaliuresis, and urinary cGMP excretion that are inhibited by efaroxan and yohimbine, implicating both, I_1-receptors and α_2-adrenoceptors in the renal effects of these drugs, and making distinction between the contributions of either receptor not easy. 2) Regardless of the receptor type, the urinary effects of moxonidine are not consistently different in SHR from WKY and SD normotensive controls, and are, therefore, not influenced by hypertension *per se*. 3) Renal responses to moxonidine are associated with elevated plasma ANP, and are 4) dose-dependently inhibited by the natriuretic peptide antagonist. These studies show, for the first time, that natriuretic peptides mediate the renal effects of acute moxonidine treatment.

Several groups have reported that acute intravenous injections of moxonidine in normotensive and hypertensive rats evoke diuresis and natriuresis (Mukaddam-Daher & Gutkowska, 2000; 1999; Allan et al., 1993; Hohage et al., 1997a,b). These actions may be mediated centrally and peripherally (Smyth & Penner, 1998). Intravenous moxonidine crosses the blood brain barrier to act preferentially on imidazoline receptors in the brainstem rostroventrolateral medulla (RVLM) (Haxiu et al., 1994), although an effect on α_2-adrenoceptors cannot be ruled out. Activation of both receptor types inhibits central sympathetic output to the peripheral vasculature, the heart and kidneys. Inhibition of renal sympathetic nerve activity leads to diuresis and natriuresis, by modulating renin release, sodium reabsorption or renal hemodynamics (Dibona, 2002). Activation of α_2-adrenoceptors in the RVLM can promote urinary sodium excretion by a renal nerve-dependent mechanism and increase urine flow rate by a pathway that involves vasopressin secretion from the paraventricular nucleus (PVN) of the hypothalamus (Menegaz et al., 2001). Activation of α_2-adrenoceptors in micturition centres of the lumbosacral and supraspinal regions leads to bladder hyperactivity (Kontani et al., 2000).

Imidazolines may also directly act on imidazoline receptors and/or α_2-adrenoceptors present in the kidney cortex and outer medulla (Li & Smyth 1993a; Greven & von

Bronewski-Schwarzer, 2001; Bidet et al., 1990, Limon et al., 1992). Selective activation of renal I_1-receptors by intrarenal infusion of moxonidine markedly increases the urine flow rate and sodium excretion (Allan et al., 1993) by a direct tubular effect (Greven & von Bronewski-Schwarzer. 2001). Although moxonidine binds to renal I_1-receptors with higher affinity than to α_2-adrenoceptors, activation of α_2-adrenoceptors may inhibit vasopressin-dependent (Nielsen et al., 2002; Edwards et al., 1992) and vasopressin-independent (Junaid et al., 1999) aquaporin-mediated water reabsorption, or stimulate local nitric oxide release in the renal medulla (Zou & Cowley, 2000).

Alternatively, Smyth et al. (2003) recently reported that low-dose moxonidine infused directly into the left renal artery resulted in similar levels of urine and sodium excretion from the left and the right kidneys, and accordingly suggested that an extra-renal diuretic and natriuretic factor mediated its renal effects. The present study shows that acute intravenous moxonidine in conscious rats increases plasma natriuretic peptides and urinary cGMP excretion. These renal effects are inhibited by anantin, a natriuretic peptide antagonist, providing clear evidence that the extra-renal factors, proposed by Smyth et al. (2003) are natriuretic peptides. The present findings substantiate the hypothesis that regardless of the receptor type involved, intravenous moxonidine injections result in elevated levels of circulating natriuretic peptides, which would act on their receptors to stimulate diuresis and natriuresis.

Natriuretic peptides participate in cardiovascular regulation through direct vasodilating and renal effects as well as by influencing sympathetic nerve activity and heart rate (Jamison et al., 1992; Melo et al., 2000; de Bold et al., 2001; Imaizumi & Takeshita, 1993). Intravenous administration of ANP in rats results in suppression of efferent activity in adrenal, renal and splenic sympathetic nerve fibers, and the effect is absent in decerebrated rats, indicating that circulating ANP modulates autonomic outflows through hypothalamic neurons that lack a blood-brain barrier (Nijima, 1989). Also, circulating natriuretic peptides act on the kidney to cause diuresis and natriuresis by stimulating the glomerular filtration rate and renal blood flow, exerting direct actions on renal proximal tubules and inner medullary collecting duct cells to inhibit sodium and water reabsorption, and by inhibiting renin and vasopressin release and aldosterone synthesis

and secretion (Jamison et al., 1992; Melo et al., 2000; de Bold et al., 2001; Imaizumi & Takeshita, 1993).

In the present study, antagonism of natriuretic peptides resulted in complete inhibition of moxonidine-stimulated urinary parameters. Although 3 natriuretic peptide receptor subtypes (NPR-A, NPR-B and NPR-C) are present in the kidney (Jamison et al., 1992), NPR-A (also known as GC-A) was shown to be the receptor subtype that mediates the acute diuretic and natriuretic effects of the cardiac natriuretic peptides ANP and brain natriuretic peptide (BNP). In NPR-A knockout mice, rapid volume expansion, a primary stimulus of natriuretic peptides (ANP & BNP) release, fails to stimulate water and sodium excretion (Kishimoto et al., 1996). Therefore, we may propose that natriuretic peptides mediate the effects of moxonidine, most likely through their NPR-A. In fact, it would be interesting, at this point, to study the effects of moxonidine in NPR-A null mice.

Investigating whether the renal effects of moxonidine are altered in hypertension, the present experiments demonstrate that the responses in SHR were not consistently different from those in two normotensive control strains. Intriguingly, differences were more influenced by strain than pressure. Moxonidine-stimulated diuresis was lower in SHR compared to normotensive SD and WKY rats. On the other hand, natriuresis and kaliuresis were lower in SHR than in SD but not in WKY rats.

The lack of stimulated renal effects by moxonidine in SHR is contrary to the expectation that inhibition of hypertension-associated renal sympathetic overactivity, which tends to promote sodium and water retention to a greater extent than in normotensive rats (Roman & Cowley, 1985), may result in enhanced renal responses to moxonidine.

Furthermore, plasma natriuretic peptide levels, which were already higher in SHR than in WKY and SD rats, were further elevated by moxonidine, but the increase in circulating levels was not reflected by the urinary parameters. This is not surprising, however, because diuresis and natriuresis are the net product of multiple hemodynamic, neural, hormonal and local factors that may be altered in hypertension (Li & Smyth, 1993b; Dibona, 2002), including renal I_1-receptors, α_2-adrenoceptors, and natriuretic peptide

receptors. Previous studies have shown that the density of renal α_2-adrenoceptors is elevated in SHR (Stanko & Smyth, 1991), but not in 1K1C hypertensive rats (Li & Smyth, 1993a). Idazoxan-labelled imidazoline receptor binding is lower in 1K1C hypertensive rat kidneys compared to sham controls (Li & Smyth, 1993a). On the other hand, renal natriuretic peptide receptors in SHR are up-regulated in the inner medulla (Guillaume et al., 1997), and are either unchanged (Tremblay et al., 1993) or reduced in glomeruli (Guillaume et al., 1997). Therefore, renal receptor regulation may counter-balance hormonal levels.

The reduced renal responses to moxonidine in SHR may also be explained by a greater drop in blood pressure in SHR following treatment. We have shown in previous studies that injections of 50 μg moxonidine in normotensive SD rats do not significantly reduce blood pressures (diastolic, systolic and mean), measured by radiotelemetry (Mukaddam-Daher & Gutkowska, 2000). Also, 50 μg moxonidine, which only slightly decreased systolic blood pressure measured by the tail-cuff method 30 min after injection in WKY rats (~10 mmHg), resulted in a significant (~40 mmHg) decrease in SHR (Mukaddam-Daher & Gutkowska, 1999).

However, although the drop in blood pressure may explain, in part, the diuretic effect of moxonidine, it may not explain the natriuretic effect, which was not different between SHR and WKY. Most importantly, whereas characterization of the receptor type mediating the renal effects of moxonidine in these conditions is not conclusive, this study proves, for the first time, that natriuretic peptides are directly involved in the renal actions of acute intravenous moxonidine in conscious rats, and that its actions are not altered by hypertension.

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3.7-FIGURE LEGENDS

Figure 1. Effect of increasing doses of clonidine on urine output (UV, ml h^{-1} $100g^{-1}$), sodium (UNaV, μmol h^{-1} $100g^{-1}$), potassium (UKV,□ μmol h^{-1} $100g^{-1}$) and cGMP (UcGMP, nmol h^{-1} $100g^{-1}$) excretions during the first hour of drug administration in Sprague-Dawley (SD), Wistar-Kyoto (WKY) and SHR (n = 5-32 rats per group per treatment). *P<0.001 vs corresponding saline control.

Figure 2. Effect of increasing doses of moxonidine on urine output, sodium, potassium and cGMP excretions during the first hour of drug administration in SD, WKY and SHR (n= 5-30 rats per group per treatment). *P<0.001 vs corresponding saline control.

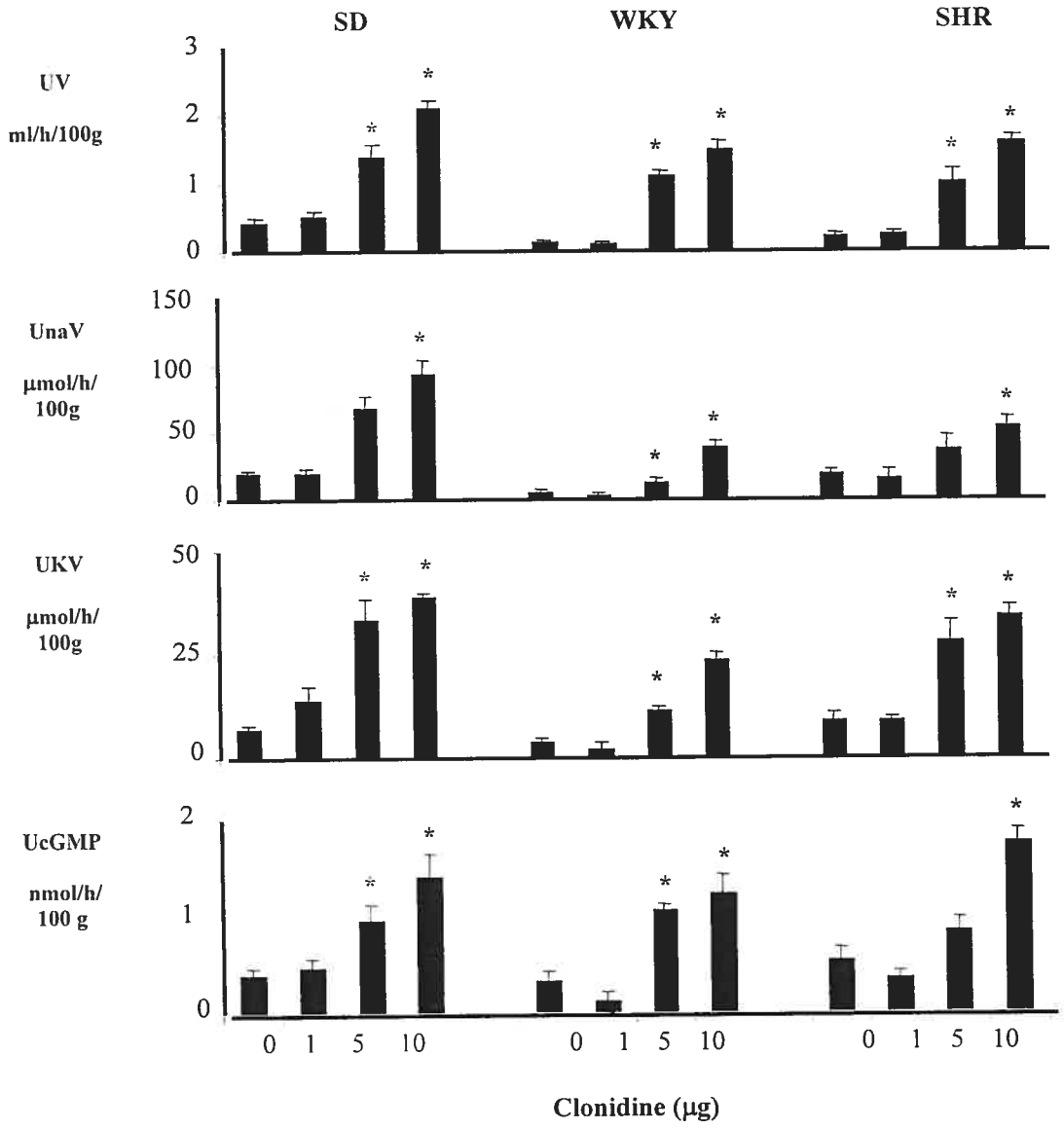
Figure 3. Urine output, sodium, potassium and cGMP excretions during the first hour of treatment with clonidine with and without pretreatment with yohimbine and efaroxan in SD, WKY and SHR (n=8-26 rats per group per treatment). *P<0.001 vs corresponding saline control. **P<0.01 vs corresponding clonidine.

Figure 4. Urine output, sodium, potassium and cGMP excretions during the first hour of treatment with moxonidine with and without pretreatment with yohimbine and efaroxan in SD, WKY and SHR (n=8-26 rats per group per treatment). *P<0.001 vs corresponding saline control. **P<0.01 vs corresponding moxonidine.

Figure 5. Urine output, sodium, potassium and cGMP excretions during the first hour of treatment with anantin, administered 10 min before 100 μg moxonidine injection in SD, WKY and SHR (n=5-26 rats per group per treatment). *P<0.001 vs corresponding saline control. **P<0.001 vs corresponding moxonidine; $^{\#}$P<0.05 vs corresponding SD and WKY; $^{\$}$P<0.05 vs corresponding SD.

Figure 6. Effect of moxonidine on plasma ANP levels 15-20 min after moxonidine injection in SD, WKY and SHR (n=5-18 rats per group per treatment). *P<0.01 vs corresponding saline control; $^{\$}$P<0.001 vs corresponding SD.

Figure 1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WKY
SHR
UV
ml/h/100g
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UnaV
µmol/h/
100g
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UKV
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UcGMP
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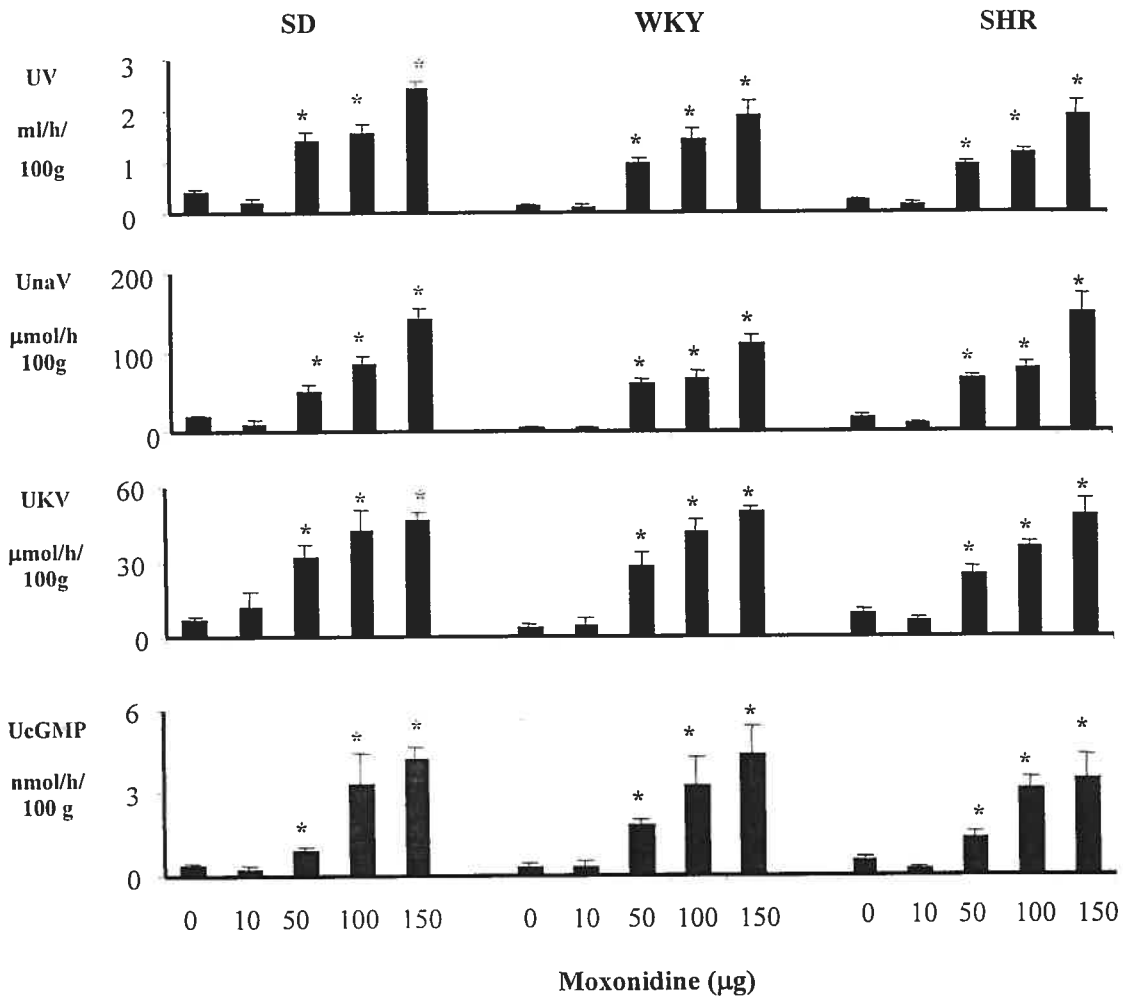
0 1 5 10
Clonidine (µg)

Figure 2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100g
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200
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UKV
µmol/h/
100g
60
30
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UcGMP
nmol/h/
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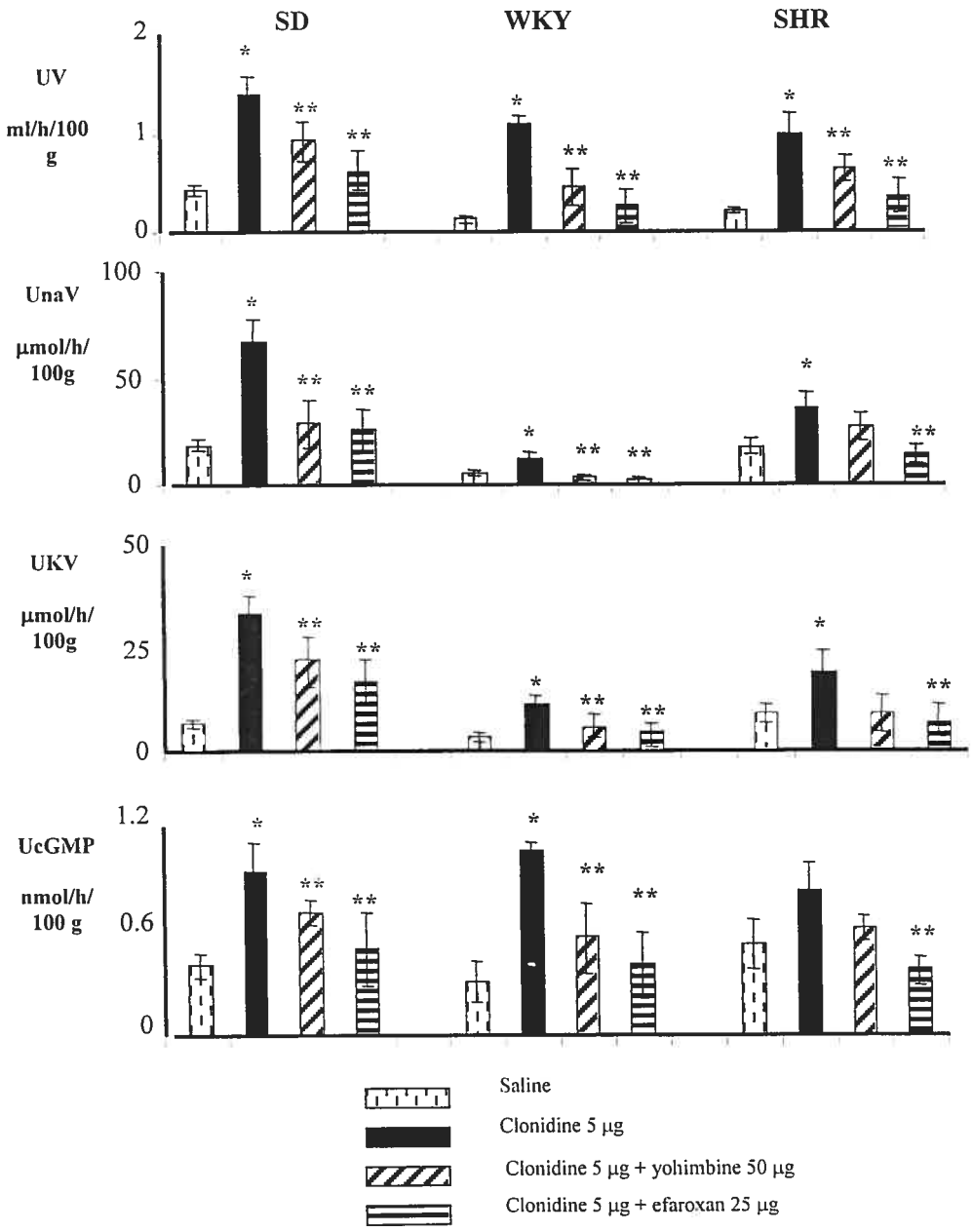
Moxonidine (µg)

Figure 3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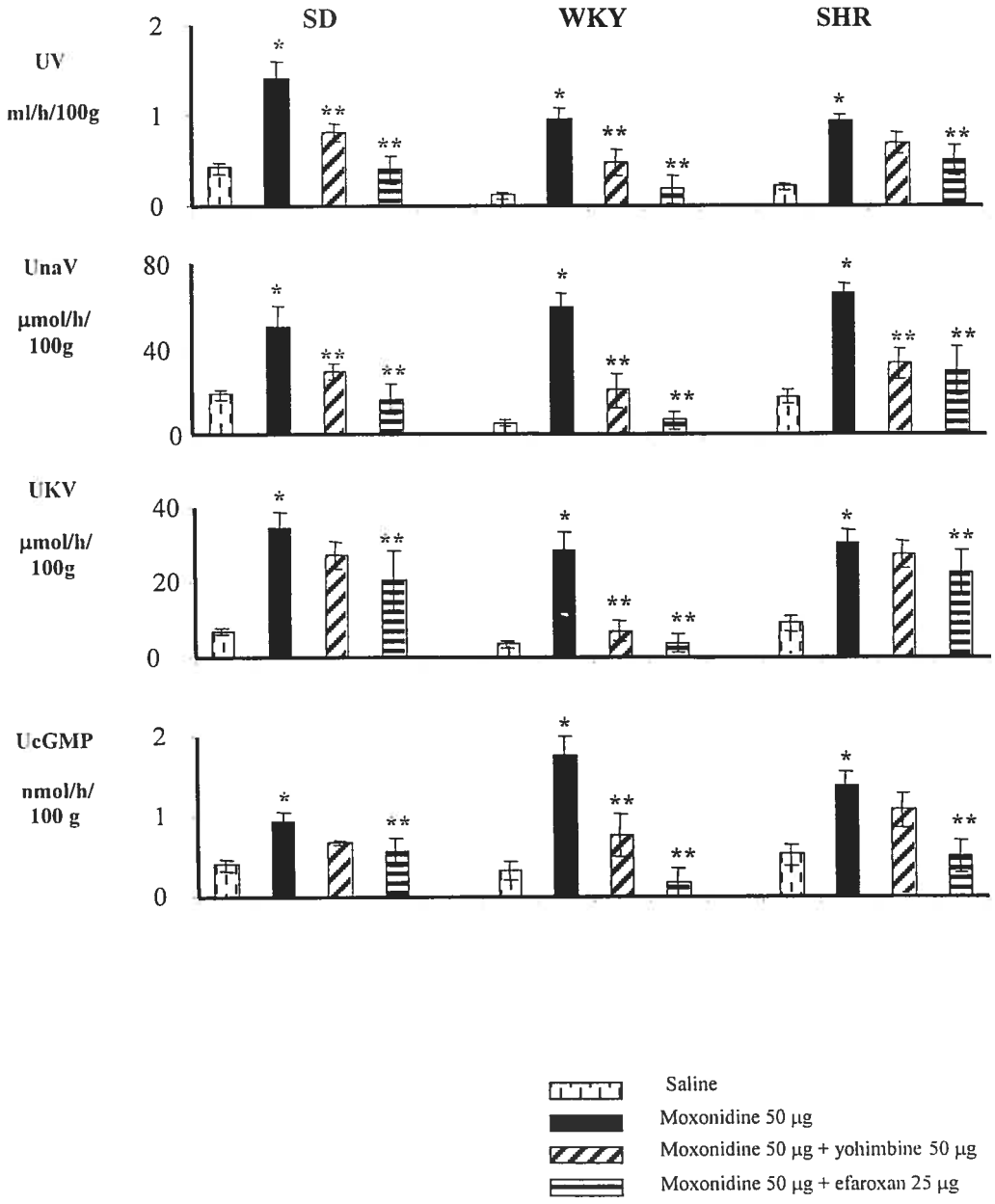
UKV
µmol/h/ 100g
50
25
0
UcGMP
nmol/h/ 100 g
1.2
0.6
0
Saline
Clonidine 5 µg
Clonidine 5 µg + yohimbine 50 µg
Clonidine 5 µg + efaroxan 25 µg

Figure 4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0
UcGMP
nmol/h/
100 g
2
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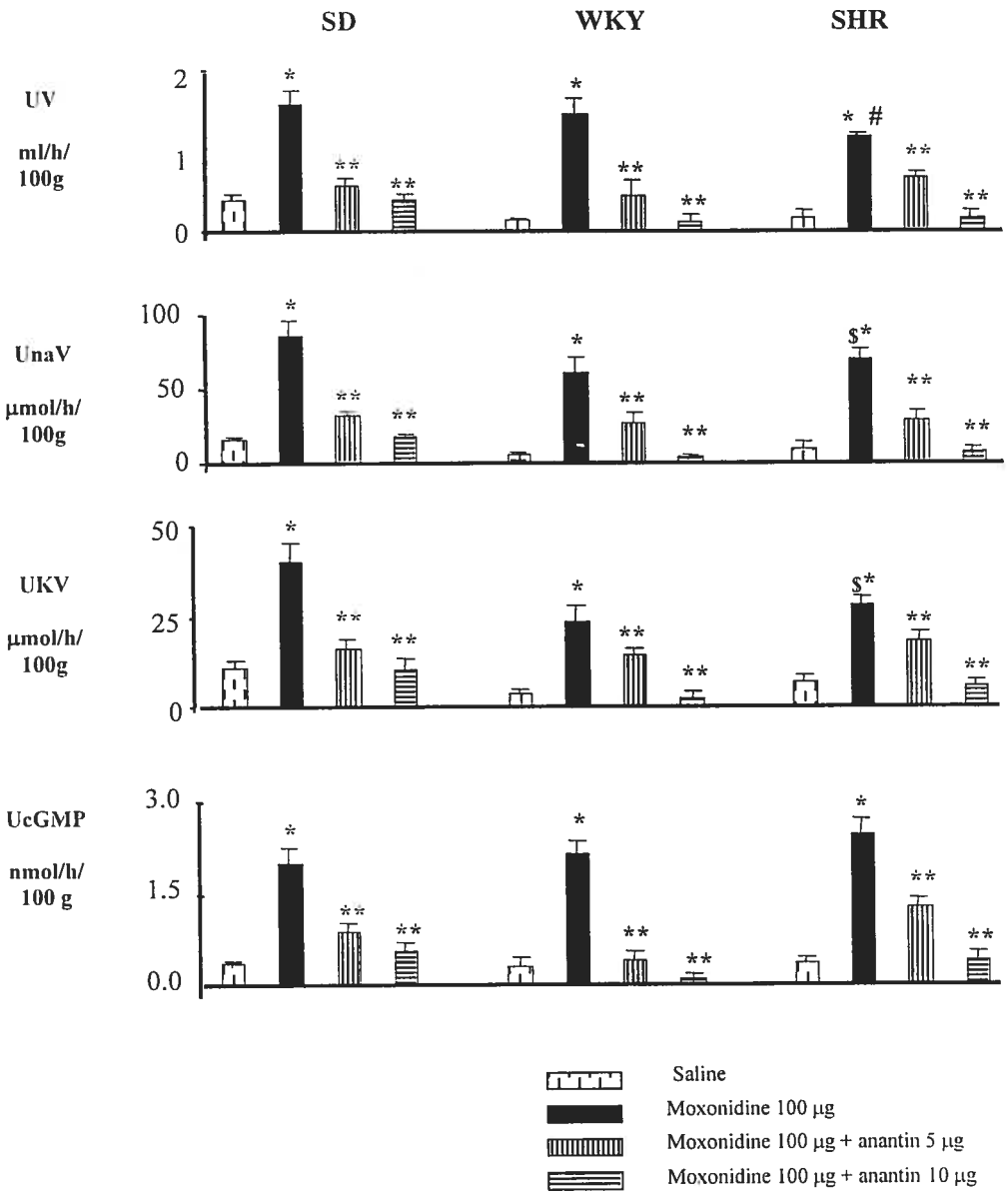
**
Saline
Moxonidine 50 µg
Moxonidine 50 µg + yohimbine 50 µg
Moxonidine 50 µg + efaroxan 25 µg

Figure 5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100g
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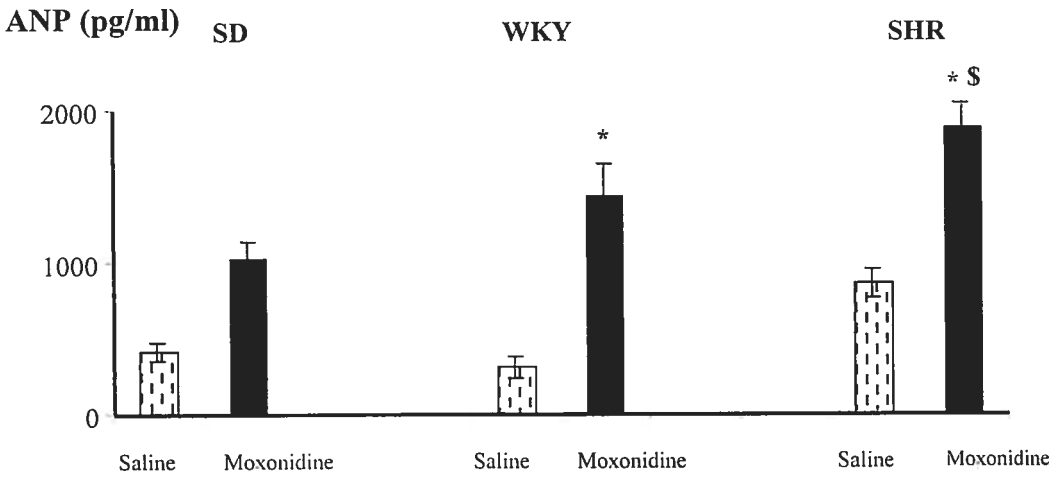
UKV
µmol/h/
100g
50
25
0
UcGMP
nmol/h/
100 g
3.0
1.5
0.0
Saline
Moxonidine 100 µg
Moxonidine 100 µg + anantin 5 µg
Moxonidine 100 µg + anantin 10 µg

Figure 6


ANP (pg/ml)
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WKY
SHR
* $
*
2000
1000
0
Saline
Moxonidine
Saline
Moxonidine
Saline
Moxonidine

CHAPITRE 4

les récepteurs aux imidazolines I_1 sont identifiés dans le cerveau, plus concentrés dans la RVLM, le centre impliqué dans la régulation de la pression artérielle. Ces récepteurs sont aussi localisés dans les reins et les médullosurrénales. Cependant, cette étude illustre pour la première fois la découverte des récepteurs I_1 dans le cœur.

Les récepteurs aux imidazolines I_1 ont été identifiés dans le cœur par plusieurs techniques comme l'autoradiographie sur les sections gelées et les membranes cardiaques en utilisant l'analogue radiomarqué de la clonidine (^{125}I-PIC) et aussi par le transfert Western et l'immunohistochimie, en utilisant un anticorps spécifique qui reconnaît les récepteurs I_1. Cette étude démontre aussi que les récepteurs I_1 ont été surexprimés dans les oreillettes des SHR, dans les ventricules des hamsters cardiomyopathiques et dans les ventricules défaillants humains.

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CHAPITRE 4

IMIDAZOLINE RECEPTORS IN THE HEART CHARACTERIZATION, DISTRIBUTION, AND REGULATION

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El-Ayoubi et al: Heart Imidazoline Receptors

Index words: Alpha2 adrenergic receptors, paraiodoclonidine, moxonidine, membrane binding, autoradiography, immunolocalization, hypertension, heart failure,

4.1-ABSTRACT

Imidazoline receptors were identified in cardiac tissues of various species. Imidazoline receptors were immunolocalized in the rat heart. Membrane binding and autoradiography on frozen heart sections using 0.5 nM para-iodoclonidine (^{125}I-PIC) revealed that binding was equally and concentration-dependently inhibited by epinephrine and imidazole-4-acetic acid (IAA), implying ^{125}I-PIC binding to cardiac α2-adrenergic and I_1-receptors, respectively. After irreversible blockade of α2-adrenergic receptors, binding was inhibited by the selective I_1-agonist, moxonidine, and the I_1-antagonist, efaroxan, in a concentration-dependent (10^{-12} to 10^{-5} M) manner. Calculation of kinetic parameters revealed that in canine left and right atria, I_1-receptor Bmax was 13.4±1.7 and 20.1±3.0 fmol/mg protein, respectively. Compared to age-matched normotensive Wistar Kyoto rats, I_1-receptors were increased in 12 week old hypertensive rat (SHR) right (22.6±0.3 to 43.7±4.4 fmol/unit area, $P<0.01$) and left atria (13.3±0.6 to 30.2±4.1 fmol/unit area, $P<0.01$). Also, compared to corresponding normal controls, Bmax was increased in hearts of hamsters with advanced cardiomyopathy (13.9±0.4 to. 26.0±2.3 fmol/unit area, $P<0.01$) and in human ventricles with heart failure (12.6±1.3 to 35.5±2.9 fmol/mg protein, $P<0.003$). These studies demonstrate that the heart possesses imidazoline I_1-receptors that are up-regulated in the presence of hypertension or heart failure, which would suggest their involvement in cardiovascular regulation.

4.2-INTROCTION

Imidazoline antihypertensive drugs that were originally thought to be α_2 adrenergic agonists also bind to non-adrenergic sites that have been named imidazoline sites. These sites are distinct from adrenergic and histaminergic sites as they show low affinity for the catecholamines epinephrine and norepinephrine as well as histamine (1-3). Imidazoline binding sites are now considered to be receptors since they fulfil the criteria for identification as receptors, including specificity of binding, association with physiological function and having endogenous ligand(s) (4). Imidazoline receptor subtypes have been defined according to their ligand affinity. Binding sites named I_1 display higher affinity for ^{3}H-clonidine or clonidine analogs, whereas I_2 sites show a 100 fold lower affinity for clonidine, but high affinity for ^{3}H-idazoxan (5, 6). I_1 sites are present in brainstem, kidney, adrenal chromaffin cells and carotid body (7-9) and have been directly associated with a regulation of vasomotor tone and the hypotensive mechanism of action of imidazoline drugs such as clonidine and moxonidine (1,10,11).

We have previously shown that in the normotensive conscious rat, activation of imidazoline receptors by acute intravenous administration of moxonidine, a highly selective imidazoline receptor agonist, is associated with increased plasma atrial natriuretic peptide (ANP) and urinary cGMP, the index of ANP activity. The effects of moxonidine are totally inhibited by efaroxan, a selective imidazoline I_1-receptor antagonist and partially by yohimbine, an α2-adrenergic receptor antagonist (12). The magnitude of moxonidine effects was altered in hypertesive rats (13). Since the heart is the major site of ANP production, these studies led us to propose that imidazoline I_1-receptors are present in the heart, and that they may be regulated in cardiovascular diseases.

Therefore, studies were performed to identify, characterize and localize I_1-receptors in the normal heart, and to investigate possible regulation in hypertension and heart failure, two diseases characterized by stimulated sympathetic efferent neuronal activity.

4.3-METHODS

Cardiac receptors were identified in normotensive rats, hamsters, dogs, sheep and humans. Receptor regulation was investigated in spontaneously hypertensive rats (SHR, 12 weeks old) in comparison to age-matched normotensive Wistar Kyoto (WKY) rats; and in normal Golden Syrian hamsters and hamsters with advanced cardiomyopathy (age >250 days). Rats and hamsters were purchased from Charles River (St. Constant, QC). Human ventricles (normal and heart failure of unknown etiology) were obtained from a tissue bank. Sheep and dog heart tissues were kindly supplied by other laboratories.

Experiments were performed according to the Canadian Guidelines. Animals were housed in temperature- and light- controlled room with food and water *ad libitum*, and maintained for at least 3 days before experimentation. After animal sacrifice, hearts were excised, flash frozen in liquid nitrogen and stored at –80°C.

Preparation of heart membranes

Heart membranes were prepared as previously described (14). In brief, dog, sheep and human heart tissues were homogenized with a polytron at 4°C in ice-cold homogenization buffer (50 mM Tris-HCl, pH 7.7, sucrose 10.27%, 5 mM EDTA, 5 mM EGTA, 100 μM phenanthroline, 50 μM phenylmethylsulfonyl fluoride (PMSF)). After centrifugation at 2000 g for 5 min, 4°C, supernatants were collected and centrifuged at 30,000 g, 4°C for 35 min. Pellets were washed with Tris-HCl (50 mM pH 7.7) containing 5 mM EDTA, then centrifuged. The pellets were re-suspended in Tris-HCl buffer (50 mM, pH 7.7) and divided into 2 fractions: one fraction was aliquoted and frozen in liquid nitrogen and the other incubated at room temperature for 35 min with ethylmaleimide (0.5 mM) (inactivates α_2-adrenergic binding) and phenoxybenzamine (1 μM) (α-adrenergic alkylating agent), both prepared in Tris-HCl buffer, pH 7.7, as described by Ernsberger et al (11). The homogenates were centrifuged at 30,000 g, 4°C for 35 min, then the pellets were washed with 50 mM Tris-HCl buffer (pH 7.7) and re-

centrifuged. The pellets were suspended in 50 mM Tris-HCl buffer (pH 7.7), aliquoted, then flash frozen in liquid nitrogen and stored at –80°C until use.

Protein content was measured spectrophotometrically using BSA as standard, according to the method of Bradford.

Membrane Binding assays

Optimal binding conditions (amount of radiolabelled ligand, membrane protein concentration and incubation time) were determined in preliminary studies according to Ernsberger et al (11). Membranes (200 μg) were incubated with ^{125}I-PIC (100,000 cpm/50 μl) and 100 μl drug or vehicle, in a total volume of 250 μl for 1 h at room temperature. The binding buffer consisted of 50 mM Tris-HCl, pH 7.7, 5 mM EDTA, 5 mM EGTA, 0.5 mM $MgCl_2$ and 50 μM PMSF. Specificity of binding was determined by increasing concentrations (10^{-12} to 10^{-5} M) of inhibiting drugs: moxonidine, (-)epinephrine, efaroxan, imidazole-4-acetic acid (IAA), prazosin, rauwolscine. The reaction was stopped by the addition of ice-cold Tris-HCl binding buffer, and rapid vacuum filtration on GF/C filters pre-soaked overnight in 0.1% polyethyleneimine (PEI). The filters were washed 2 times with 3 ml ice cold binding buffer, dried and counted. Binding in the presence of 10 μM piperoxan was considered nonspecific (NSB).

Autoradiography

Rat and hamster hearts were rapidly isolated after decapitation, and heart chambers separated and snap-frozen in prechilled isopentane. Cryostat whole heart sections (20 μm) from hamsters and 4 chambers of the rat heart were cut and mounted on acid-washed gelatinized slides, then placed overnight in a partial vaccum at –4°C. Slides were stored in boxes with Drierite at –80°C until the autoradiographic procedures were performed.

Autoradiography was performed as previously described (15), using the same conditions determined above for membrane binding. Duplicate slides were brought to room temperature in pre-incubation buffer containing 50 mM Tris-

HCl, pH 7.7, and 0.1% PEI for 15 minutes to reduce non specific binding. The slides were then incubated with the ^{125}I-PIC prepared in binding buffer, for 1 h at room temperature. The incubation buffer consisted of 50 mM Tris-HCl, pH 7.7, 5 mM EDTA, 5 mM EGTA, 0.5 mM $MgCl_2$ and 50 μM PMSF. Characterization of heart receptors was performed by binding of ^{125}I-PIC to adjacent sections under identical incubation conditions except for the irreversible inhibition of adrenergic receptors by incubating the sections for 35 min in 50 mM Tris-HCl (pH 7.7) in the presence of phenoxybenzamine (1 μM) and ethylmaleimide (0.5 mM). NSB was determined in the presence of 10 μM piperoxan.

Tissue sections were washed for two min each, with ice-cold incubation buffer, followed by 2 washes in 50 mM Tris-HCl buffer, pH 7.7, at 4°C and finally dipped in distilled water to wash out salts. The slides were dried under a stream of cold air. The dried tissue sections were exposed in phosphor-sensitive cassette for 48 h, then scanned, visualized and quantified by PhosphorImager (Image Quant, Molecular Dynamics, Sunnyvale CA).

Immunolocalization

Hearts were immediately excised, washed in fresh ice-cold phosphate buffered saline (PBS), then fixed in 4% paraformaldehyde for 18 h at 4°C. After washing in PBS twice and overnight incubation in 0.5 M sucrose in PBS, the hearts were frozen in prechilled isopentane then stored at -80°C until processed. Cryostat whole heart sections, 6-7 μm thick, were cut and mounted on poly-L-lysine coated glass slides and placed overnight in a partial vacuum at 4°C.

Immunhistochemistry was performed as follows: slides were treated with 95% methanol/0.3% H_2O_2 for 30 min at 22°C, washed in PBS and preincubated with 1% BSA-PBS for 30 min, followed by incubation with I-receptor antibody or with nonimmune serum (donated by the Late Dr. D. Reis, Weill Medical College of Cornell University, NY) for the control sections. After washing with PBS, the sections were incubated with F(ab')2 fragments of horseradish peroxidase-

conjugated goat anti-rabbit IgG at a dilution of 1:100 for 1 h at room temperature. The sections were reacted in 0.05% diaminobenzidine in 0.05 M Tris-HCl buffer (pH 7.4) in the presence of H_2O_2, then faintly counterstained with hematoxylin for light microscopy observation.

Immunoblotting

Immunoblotting was performed on cardiac tissues and compared to brainstem, used as a positive control, being the major site of imidazoline receptors. Denatured membrane protein samples (80 μg) were electrophoresed on 10% polyacrylamide gels followed by transfer to nitrocellulose (Hybond-P; Amersham, Arlington Heights, IL). The membranes were incubated for 1 h with anti-imidazoline antiserum diluted 1:1000.

Blots were washed then incubated for 2 h with horseradish peroxidase-conjugated anti-rabbit IgG antiserum diluted 1:5000 in Tris-buffered saline and 5% milk. Immunoreactive bands were visualized by developing on film for 3 min as recommended by Amersham's enhanced chemiluminescence (ECL) detection system (Amersham ECL Hyperfilm).

Drugs

^{125}I-para-iodoclonidine (2200 Ci/mmol) was obtained from New England Nuclear (Boston, MA) and stored at –20°C in ethanol. Moxonidine (generously donated by Solvay Pharmaceuticals, Hannover) was dissolved in 0.1 M acetic acid. Efaroxan, rauwolscine, prazosin, imidazole-4-acetic acid, phenoxybenzamine, ethylmaleimide, anandamide, and piperoxan (Sigma, St. Louis, MA, USA) were dissolved in water or ethanol as required; and (-) epinephrine was prepared in 0.001% ascorbic acid. All compounds were prepared daily and diluted in binding buffer to the required concentrations immediately before assay.

Data analysis

The equilibrium dissociation constant (Kd) and maximum binding capacity (Bmax) for the ligands used in the competitive binding radioreceptor studies were

calculated by the iterative computerized non-linear curve fitting method using the LIGAND computer program (Elsevier-Biosoft, Cambridge, UK). Data from human and hamster heart failure and hypertensive rats were compared to corresponding normal controls using the unpaired Student's t-test. Statistical significance was taken as $p<0.05$. All data are reported as mean ± SEM.

4.4-RESULTS

The presence of imidazoline receptors in the heart was shown by immunohistochemistry, Western blot, membrane binding and autoradiography. Immunolocalization of imidazoline receptors to normal rat heart was identified by the strong yellow staining observed in atria as compared to the non-immune serum (Figure 1) and faint staining in rat ventricles (not shown). Western blot analysis of rat brainsetm, dog and human heart membranes using a specific anti-imidazoline receptor protein antiserum showed the presence of at least five immunoreactive imidazoline receptor protein bands. The apparent molecular masses of these peptides were ~30, ~46, ~66, ~90 and ~150-200 (double band) KDa (Figure 2). The strongest bands were observed in rat brainstem>human ventricles>dog ventricles>dog atria.

Membrane Binding

The radiolabelled ligand ^{125}I-PIC binds with high affinity to both α_2-adrenergic and imidazoline I_1-receptors in brain, platelets and carotid bodies (16-19). Binding of ^{125}I-PIC to the heart membranes was rapid, specific, saturable and of high affinity. Nonspecific binding determined in the presence of piperoxan was less than 5%. Figure 3 illustrates curves plotted from data of binding assays of dog left atrial membranes represented as %B/Bo, where B and Bo represent respectively, specific binding with and without competing drugs. In left atria, bound ^{125}I-PIC was progressively inhibited by increasing drug concentrations, so that at 10^{-5} M inhibition by moxonidine (imidazoline I_1-receptor agonist) represented 70%; epinephrine (α2 agonists) 44%; rauwolscine (non-imidazoline α2 antagonists), 47%; and only 20% by prazosin (α1-antagonist).

Similar inhibition profiles were obtained in ventricles, where binding was also inhibited by efaroxan (imidazoline receptor antagonist) and represented 50%. These results are consistent with ^{125}I-PIC binding to imidazoline I_1 and α_2-adrenergic, but not α1-adrenergic receptors in the heart. After irreversible blockade of α-adrenergic receptors, epinephrine and rauwolscine only minimally

inhibited binding (range of 0 to 20%), confirming blockade of α_2-adrenergic receptors in the membrane preparations. On the other hand, moxonidine inhibited binding in a concentration-dependent manner, reaching total inhibition at 10^{-6} and 10^{-5} M concentrations (Figure 3).

Heart imidazoline receptor kinetic parameters were calculated from curves obtained by moxonidine inhibition of binding in the absence of α_2-adrenergic receptors. Table 1 shows that dog left and right atrial membranes exhibited similar binding capacity, where Bmax represented 13.4±1.7 and 20.1±3.0 fmol/mg protein, with Kd=3.9±1.0 and 9.2±1.4 nM, respectively. Imidazoline receptor binding was similar in left and right ventricular membranes (Bmax= 3.7±0.6 and 5.0±0.4 fmol/mg protein, respectively), but lower than those in atria. Receptor affinity was within the same nanomolar range (Kd = 2.6±0.1 vs 4.0±0.41 nM). Similarly, after α2-adrenergic receptor blockade, imidazoline binding in sheep atrial membranes Bmax was 17.5±3.5 fmol/mg protein, and Kd = 15 ±1 nM; and in membranes from normal human ventricles, Bmax was 12.6±1.3 fmol/mg protein, and Kd, 13.5±0.6 nM (Table 1).

Autoradiography

The results obtained from heart membrane binding studies were further confirmed by autoradiography performed on rat and hamster frozen heart sections. Binding of ^{125}I-PIC was equally inhibited (50%) by 10^{-6} M IAA (imidazoline I_1-receptor agonist) and 10^{-6} M epinephrine.

Furthermore, ^{125}I-PIC binding to cannabinoid receptors, previously shown to be stimulated by I_1-receptor agonists (20) was verified using increasing concentrations of anandamide, a cannabinoid receptor agonist. In these sections, anandamide did not significantly affect binding, representing only 10 and 11 % inhibition in left and right rat atria, respectively. These studies demonstrate that ^{125}I-PIC binding is mediated by imidazoline I_1-receptors and α_2-adrenergic receptors, but not cannabinoid receptors. In further studies performed on hamster

hearts after irreversible blockade of α2-adrenergic receptors, ^{125}I-PIC binding was not inhibited by epinephrine, but by moxonidine in a concentration-dependent manner, reaching 70±3% at 10^{-5} M concentrations.

Receptor Regulation

Figure 4 shows the effect of cardiovascular diseases on 125I-PIC binding to rat and hamster hearts. Total specific 125I-PIC binding to heart sections after α2-adrenergic receptor blockade increased in SHR atria and CMO hamster hearts. Inhibition of binding by 10-5 M moxonidine represented 60± 4 % of total binding in WKY atria and increased to 90±2 % in SHR atria. Compared to WKY, Bmax increased significantly (n=6, $p<0.001$) in SHR left (13.3±0.6 to 30.2±4.1 fmol/unit area) and right (22.6±0.3 to 43.7±4.4 fmol/unit area) atria, but no change occurred in ventricles.

Binding affinity (Kd) was not altered by hypertension (Table 2). In normal hamster hearts, imidazoline receptor Bmax was 13.9±0.4 fmol/unit area, and increased in cardiomyopathy to 26.0±2.3 fmol/unit area ($P<0.01$), whereas Kd of these tissues did not change (7.8±0.4 vs 11.7±1.4 nM). Similarly, imidazoline receptor total binding was increased by 3 fold in failing human heart ventricular membranes. Figure 5 illustrates curves of 125I-PIC binding to human ventricular membranes after blockade of α2-adrenergic receptors and inhibition of binding by competing drugs.

Inhibition of binding by moxonidine and IAA represented 70±4% and 51±2% respectively, in the normal human ventricles, and increased to 95±1% and 85±2% in the failing heart membranes, indicating receptor up-regulation. This up-regulation was due to an increased Bmax (12.6±1.3 to 35.5±2.9 fmol/mg protein, n=3, $P<0.003$), but not affinity.

4.5-DISCUSSION

This study consists of two main parts: identification of imidazoline receptors in the heart and receptor regulation in cardiovascular diseases. Qualitative demonstration of imidazoline receptors in the heart was achieved by immunolocalization using the polyclonal antiserum to an I-receptor binding protein isolated from bovine adrenal chromaffin cells. This protein specifically labels imidazoline receptor protein in rat brain, rat aortic VSMC, bovine pulmonary artery endothelial cells, and human brain. The antibody recognizes the ~70, ~45 and ~29 kDa imidazoline receptor subtypes but not α2-adrenergic receptors (21).

Using the same antibody, Western blot analysis of atrial and ventricular heart membranes showed multiple molecular mass peptides, similar to those thus far described in the brain but in much lower quantities. This is consistent with the finding that a partial cDNA clone detected by imidazoline receptor-selective antisera is present in the human heart, albeit in very much lower levels than those in pituitary and brain (22). However, we presently have no explanation as to the appearance of bands corresponding to high molecular mass peptides in samples from brainstem, ventricles and atria, except possible dimerization of the 85 KDa peptide, reported as the full-length imidazoline receptor protein (23).

Since the receptor antibody does not discriminate between I1- and I2-receptors, competitive membrane binding and autoradiography techniques were employed using the radiolabelled ligand, paraiodoclonidine (125I-PIC) which binds with high affinity to α2-adrenergic receptors and imidazoline I1-receptors but with very low affinity to I2-receptors (18). In these studies, identification of imidazoline I1-receptors was achieved using optimal conditions that favor imidazoline over α2-adrenergic receptor binding, such as low $MgCl_2$ buffer (24). Furthermore, binding was performed with and without blockade of adrenergic receptors, by using a combination of ethylmaleimide, which inhibits specific binding of ^{125}I-PIC to α2-adrenergic receptors, and phenoxybenzamine, which irreversibly blocks α2- and α1-adrenergic receptors at high potency, but has minimal

effects on imidazoline receptors (11, 25). Specific binding to I_1-receptors was determined using moxonidine, which shows a 100- and 700- fold selectivity for the I_1 imidazoline receptor over α2b-adrenergic receptors in rat brain and renal medullary membranes, respectively (9,18).

^{125}I-PIC binding was observed in dog heart membranes and hamster and rat heart sections. Binding was inhibited by IAA and epinephrine, implying that the heart exhibits both imidazoline I_1- and α2-adrenergic receptors, respectively. After irreversible inhibition of adrenergic receptors, binding of ^{125}I-PIC was no longer inhibited by increasing concentrations of prazosin, epinephrine and rawoulscine, confirming total inhibition of α-adrenergic receptors. On the other hand, concentration-dependent inhibition of binding with moxonidine, efaroxan and IAA indicated the presence of specific I_1-receptors, and the lack of inhibition with anandamide confirmed that ^{125}I-PIC did not bind to cannabinoid receptors that have been proposed to bind moxonidine (20).

Calculation of kinetic parameters in heart membranes from sheep, dog and human, or rat and hamster hearts revealed that the affinity of I_1-receptors in the heart is in the nanomolar range, similar to those reported for other tissues using ^{3}H-clonidine, such as bovine, rat and human brainstem (6-7 nM) (26), and bovine adrenomedullary cells (Kd 12 nM) (28). The imidazoline receptor binding capacity is 2 fold greater in atria than ventricles, but at least 10 fold lower than that reported by Ernsberger and co-workers in VLM membranes, the primary site of imidazoline binding (18).

The second aim of the study dealt with the regulation of heart I_1-receptors. Again, membrane binding or autoradiography revealed a 2-fold increase of imidazoline sites in atria of SHR, a 3-fold increase in failing human ventricles and a 2-fold increase in hearts of hamsters with advanced cardiomyopathy. These data indicate that imidazoline I_1-receptors in cardiac tissue are altered in cardiovascular diseases; and therefore, it is reasonable to suggest that heart imidazoline I_1-receptors are involved in cardiovascular regulation.

The mechanisms of heart I-receptor regulation in cardiovascular diseases are beyond the scope of this study. However, several studies have shown that imidazoline sites are subject to physiological and pharmacological regulation and that I-receptor regulation is tissue specific. Imidazoline receptor up-regulation has been reported in post-mortem brains of suicide victims (27), in platelets of patients with depression (29) and platelets of postmenopausal women (30). Chronic treatment with the antidepressant drug, imipramine, downregulates I_1-imidazoline receptors in rat brainstem (31). Chronic administration of idazoxan to rabbits causes a substantial decrease in the number of renal imidazoline binding sites (32), but increases the density of central I-receptors (33). I-receptor binding is also up-regulated in kidneys of genetically hypertensive rats (34).

Regardless of the mechanisms of regulation, up-regulated heart imidazoline receptors in cardiovascular diseases characterized by sympathetic efferent neuronal overactivity may reflect the benefits of imidazoline receptor agonists observed in patients as compared to normal subjects. Heart imidazoline receptors may play an important role in normal cardiovascular regulation, especially since agmatine, an endogenous ligand for imidazoline receptors, is present in the heart (35), and that clonidine (imidazoline I_1 and α_2-adrenergic receptor agonist) stimulates the release of ANP from isolated rat hearts (36).

ANP is a cardiac hormone, which plays an integral role in volume and pressure homeostasis in normal and pathophysiological conditions. Circulating ANP levels are increased in hypertension and heart failure; interestingly, in parallel to the present finding of up-regulated imidazoline receptors in the heart.

In conclusion, this study demonstrates the presence of imidazoline I_1-receptors in the heart, and shows that these receptors are up-regulated in cardiovascular diseases. Heart imidazoline receptors may be important in the regulation of cardiovascular function, acting locally to exert cardioprotective effects, similarly to their reported neuroprotective (21) and nephroprotective effects (37).

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Table 1: Kinetic parameters (Bmax, maximum binding capacity and Kd, dissociation constant) obtained by binding ^{125}I-PIC to atrial and ventricular membranes and inhibition of binding with increasing concentrations of moxonidine in the absence of α2-adrenergic receptors (see Methods).

	Bmax (fmol/mg protein)	Kd (nM)
Dog		
Left atria	13.4±1.7	3.9±1.0
Right atria	20.1±3.0	9.2±1.4
Left ventricles	3.7±0.6	2.6±0.1
Right ventricles	5.0±0.4	4.0±0.4
Sheep Atria	17.5 ± 3.5	15.0±1.1
Human Ventricles	12.6±1.3	13.5±0.6

Table 2: Regulation of imidazoline receptor kinetics in rat hypertension and human and hamster heart failure (see Methods). *P<0.01 vs corresponding control.

	Bmax (fmol/mg protein)	**Kd** (nM)
Rat *Left atria*		
WKY	13.3±0.6	4.8±0.4
SHR	30.2±4.1*	3.9±0.7
Rat *Right atria*		
WKY	22.6±0.3	4.5±0.3
SHR	43.7±4.4*	7.2±0.8
Hamster heart		
Normal	13.9±0.4	7.8±0.4
CMO	26.0±2.3	11.7±1.4

4.7-FIGURE LEGENDS

Figure 1: Immunolocalization of imidazoline receptors in right atria of normotensive rats

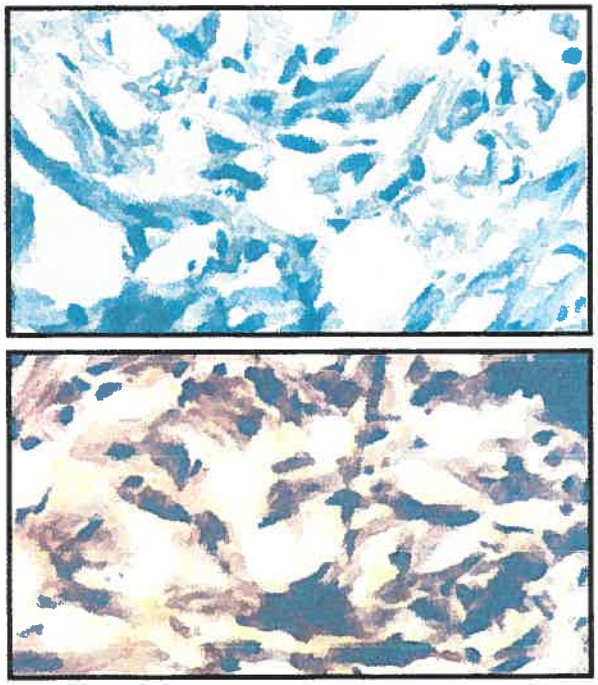
Figure 2: Immunoblot analysis of imidazoline receptor protein in rat brain, dog atria and ventricles and human ventricles

Figure 3: Binding of ^{125}I-PIC to dog left atrial membranes and inhibition of binding with α-adrenergic and imidazoline receptor agonists and antagonists. **I** = binding in the presence of α2 -adrenergic receptors; **II** = binding after irreversible blockade of α2-adrenergic receptors with ethylmaleimide and phenoxybenzamine

Figure 4: Autoradiography of ^{125}I-PIC binding in left atrial sections of hypertensive (SHR) rats vs normotensive (WKY) controls and in cardiomyopathic hamster (CMO) vs normal hamster hearts.

Figure 5: Binding of ^{125}I-PIC to human ventricular membranes obtained from normal and heart failure patients, after irreversible blockade of alpha2 adrenergic receptors with ethylmaleimide and phenoxybenzamine and inhibition of binding with moxonidine, IAA, and epinephrine.

Figure 1



Negative control

Imidazoline receptor antibody

Figure 2

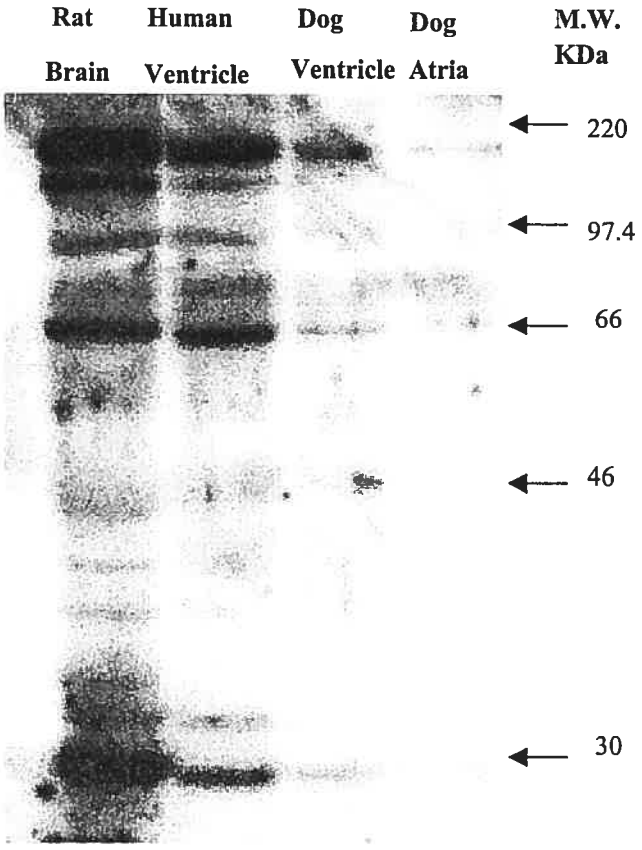

Rat Brain
Human Ventricle
Dog Ventricle
Dog Atria
M.W. KDa
220
97.4
66
46
30

Figure 3

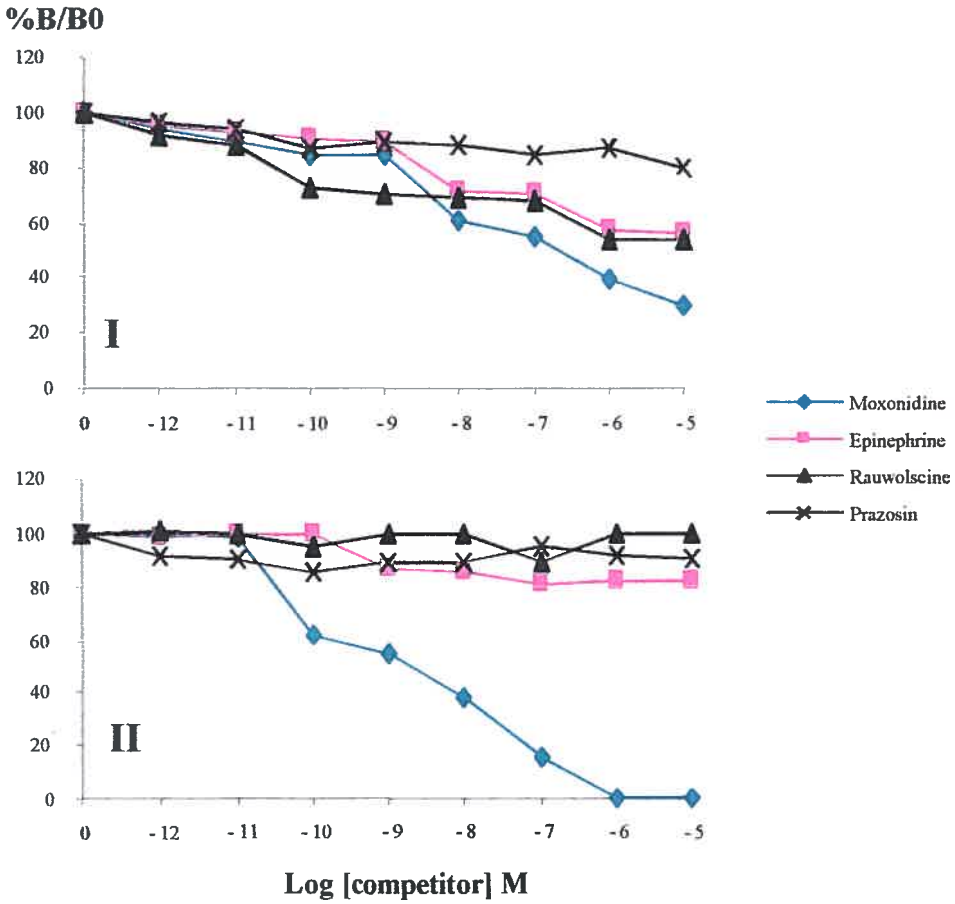

%B/B0
120
100
80
60
40
20
0
I
0
-12
-11
-10
-9
-8
-7
-6
-5
II
Moxonidine
Epinephrine
Rauwolscine
Prazosin
Log [competitor] M

Figure 4

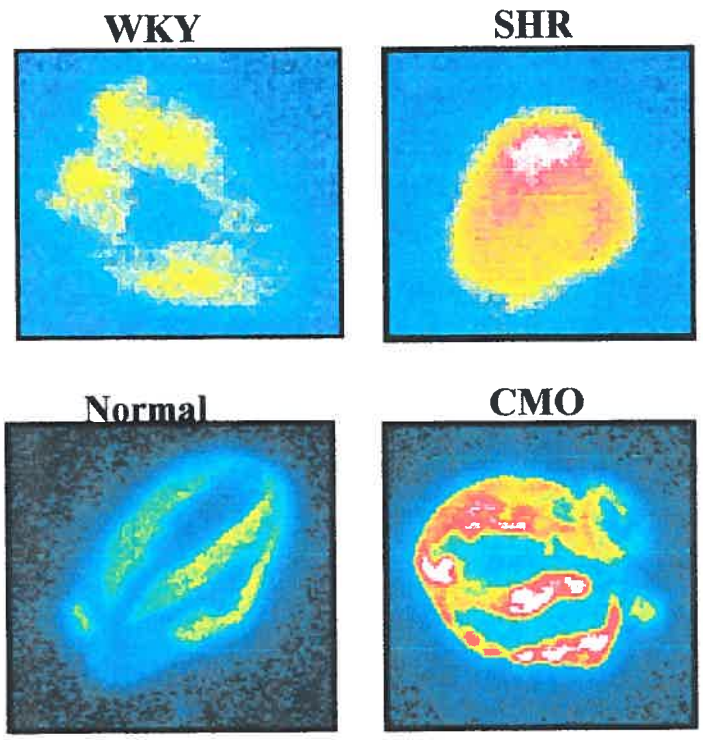
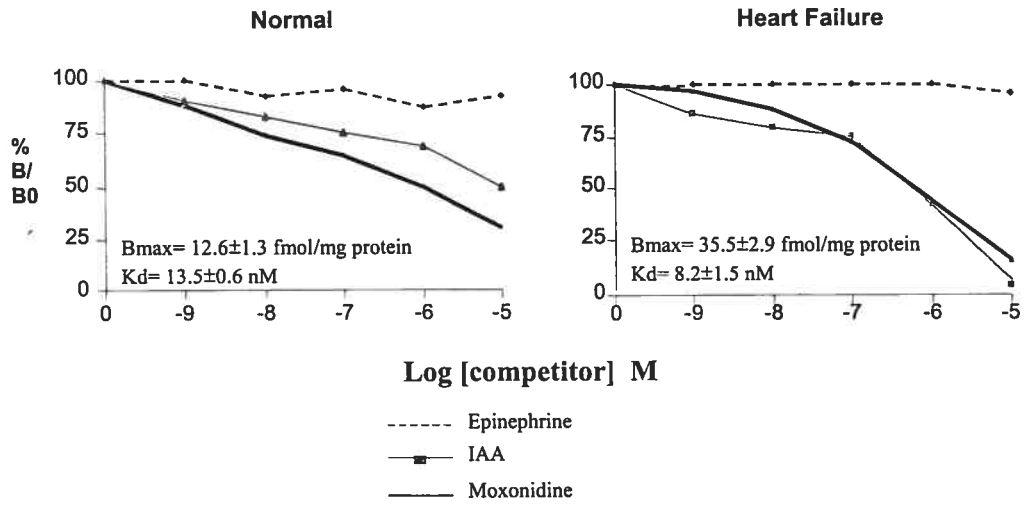


Figure 5


Normal
Heart Failure
100
75
50
25
0
% B/ B0
Bmax= 12.6±1.3 fmol/mg protein
Kd= 13.5±0.6 nM
Bmax= 35.5±2.9 fmol/mg protein
Kd= 8.2±1.5 nM
0
-9
-8
-7
-6
-5
Log [competitor] M
Epinephrine
IAA
Moxonidine

CHAPITRE 5

La densité des récepteurs I_1 étaient normalisée de façon dose-dépendante suite au traitement chronique (4 semaines) des rats SHR à la moxonidine. Cet effet était associé à une normalisation de l'expression de l'ANP dans les oreillettes des SHR.

Ann NY Acad Sci 2003

CHAPITRE 5

NORMALIZATION OF UP-REGULATED CARDIAC IMIDAZOLINE I_1-RECEPTORS AND NATRIURETIC PEPTIDES BY CHRONIC TREATMENT WITH MOXONIDINE IN SHR

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Imidazoline receptors; moxonidine; natriuretic peptides.

5.1-ABSTRACT

The effect of treatment with moxonidine (120 μg/kg/h s.c., 4 weeks) on cardiac I_1-receptors and natriuretic peptide synthesis was evaluated in SHR. I_1-receptor protein (85 kDa) was upregulated in SHR atria, and normalized in right and left atria by moxonidine. Similarly, moxonidine normalized atrial and ventricular ANPmRNA and BNPmRNA. This study shows that cardiac I_1-receptors are functional, being regulated by hypertension and by chronic exposure to agonist; and that cardiac natriuretic peptides may be regulated by I_1-receptor-mediated mechanisms.

5.2-INTRODUCTION

Imidazoline I_1-receptors are non-adrenergic and non-cholinergic neurotransmitter receptors present in the brainstem, adrenal chromaffin cells and kidneys. Activation of I_1-receptors is associated with blood pressure reduction, primarily by sympatho-inhibition (1,2). In addition, we have recently identified imidazoline I_1-receptors in the heart atria and ventricles, and shown that heart imidazoline receptors are up-regulated in cardiovascular diseases, such as hypertension and heart failure (3), implying that heart imidazoline receptors may be functional and involved in cardiovascular regulation.

Moxonidine, an antihypertensive imidazoline compound reduces blood pressure by activation of central and peripheral imidazoline I_1-receptors and subsequent decrease of sympathetic nervous activity and stimulation of renal actions (2). Acute moxonidine injections in normotensive Sprague-Dawley (SD) rats dose-dependently increase diuresis, natriuresis and cGMP excretion as well as plasma levels of natriuretic peptides (4), cardiac hormones that reduce blood pressure by several mechanisms, including vasodilation, diuresis, natriuresis and sympatho-inhibition.

Based on the findings that cardiac natriuretic peptides may be involved in the acute activation of imidazoline receptors by moxonidine (4), and that both, imidazoline receptors and natriuretic peptide synthesis are increased in hearts of hypertensive rats (3,5), the following studies were performed to investigate the effect of one-month treatment with moxonidine on imidazoline receptors and natriuretic peptide expression in hypertensive rat hearts, with the aim of demonstrating functionality of cardiac imidazoline I_1-receptors.

5.3-METHODS

Female SHR (12-14 weeks old) were treated during one month with 2 doses of moxonidine (60, & 120 μg/kg/h) or saline vehicle, via Alzet osmotic minipumps (2ML4) implanted under the neck skin. Sprague-Dawley rats (200-225 g) served as normotensive controls. Animals were housed in temperature- and light-controlled room with food and water ad libitum. After one month of treatment, the rats were sacrificed by decapitation and the hearts were rapidly isolated, divided into 4 compartments, then flash frozen in liquid N_2 and stored at –80°C.

Imidazoline receptor regulation in cardiac atria and ventricles was analyzed by Western blot using a polyclonal imidazoline receptor antibody, as we have previously described (3). Total RNA from ventricles and atria were extracted, then reverse-transcribed into cDNA and subjected to semi-quantitative PCR, using specific primers for ANP, BNP and GAPDH (5). Values normalized to corresponding GAPDH are reported as percent of normotensive controls or vehicle-treated SHR controls.

5.4-RESULTS

Immunoblotting revealed that, compared to normotensive controls (100%), the intensity of the bands that correspond to 85 kDa was increased (135±3%; n = 10; P< 0.001) in SHR atria. Compared to vehicle-treated SHR (100%), treatment with 60 & 120 μg moxonidine significantly (n = 6-10; p<0.001) decreased the intensity of the 85 kDa band in right atria to 51±2% and 47±3%, respectively. The bands that correspond to 29/30 kDa were not altered in SHR with or without treatment (Fig 1). The intensity of the bands corresponding to 29/30 and 85 kDa proteins were not increased in hypertensive rat ventricles. One-month treatment with 2 doses of moxonidine had no effect on the 85 kDa band, but was associated with reduced intensity of the bands corresponding to 29/30 kDa to 89±2% of vehicle-treated SHR (n= 6; P< 0.002).

ANP mRNA decreased in right atria and left ventricles to 68±2% and 70±6% of corresponding vehicle-treated hypertensive controls, respectively (p<0.001). Similarly, right atrial and left ventricular BNP mRNA decreased to 46±1% and 65±1% (p< 0.001) (Fig 2). The levels of ANP and BNP mRNA were not significantly different from those measured in normotensive controls.

5.5-DISCUSSION

This study shows that chronic 1-month treatment of hypertensive rats with moxonidine is associated with normalization of up-regulated imidazoline receptors and natriuretic peptide synthesis in cardiac atria and ventricles.

Imidazoline receptors of 29/30 kDa, 45 kDa, 85 kDa, 176 kDa proteins have been isolated from brainstem RVLM (6). Similar proteins are also present in the heart (3). However, it is not yet known which protein corresponds to the functional imidazoline I_1-receptor. Based on our present finding, we may suggest that functional cardiac imidazoline I_1-receptors are tissue specific, being differentially regulated in atria and ventricles by hypertension and chronic exposure to agonist. Whereas the 85 kDa protein may correspond to the functional I_1-receptor in atria, it may be the 29/30 kDa protein that is functional in ventricles. However, further studies are required to confirm this observation.

Previous studies from our lab and others have shown that natriuretic peptides synthesis is increased in hypertensive rat hearts (3,7,8). In the present study, chronic treatment of SHR with 60 & 120 μg/kg/h moxonidine, over one month, is associated with significantly reduced ANP mRNA and BNP mRNA in right atria and left ventricles as compared to corresponding vehicle-treated SHR. The mechanisms of natriuretic peptide decrease by chronic moxonidine have not been investigated, but these changes parallel the changes in imidazoline receptor proteins, both are up-regulated by hypertension and down-regulated by moxonidine treatment. Therefore, based on the present findings and on our previous results that acute moxonidine injections increase circulating ANP levels, it appears likely that cardiac natriuretic peptides may be regulated by imidazoline I_1-receptor-mediated mechanisms.

Moxonidine was generously provided by Solvay, Germany. Studies are supported by the Canadian Institutes for Health Research, and the Heart and Stroke Foundation of Canada.

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5.7-FIGURE LEGENDS

Figure 1: Western blot analysis of imidazoline receptor proteins in right atria in response to 1-month treatment with 2 doses of moxonidine.

Figure 2: Effect of moxonidine treatment on natriuretic peptides ANP and BNP mRNA in right atria of SHR. Results are normalized to corresponding GAPDH and reported as percent of vehicle-treated SHR.

Figure 1

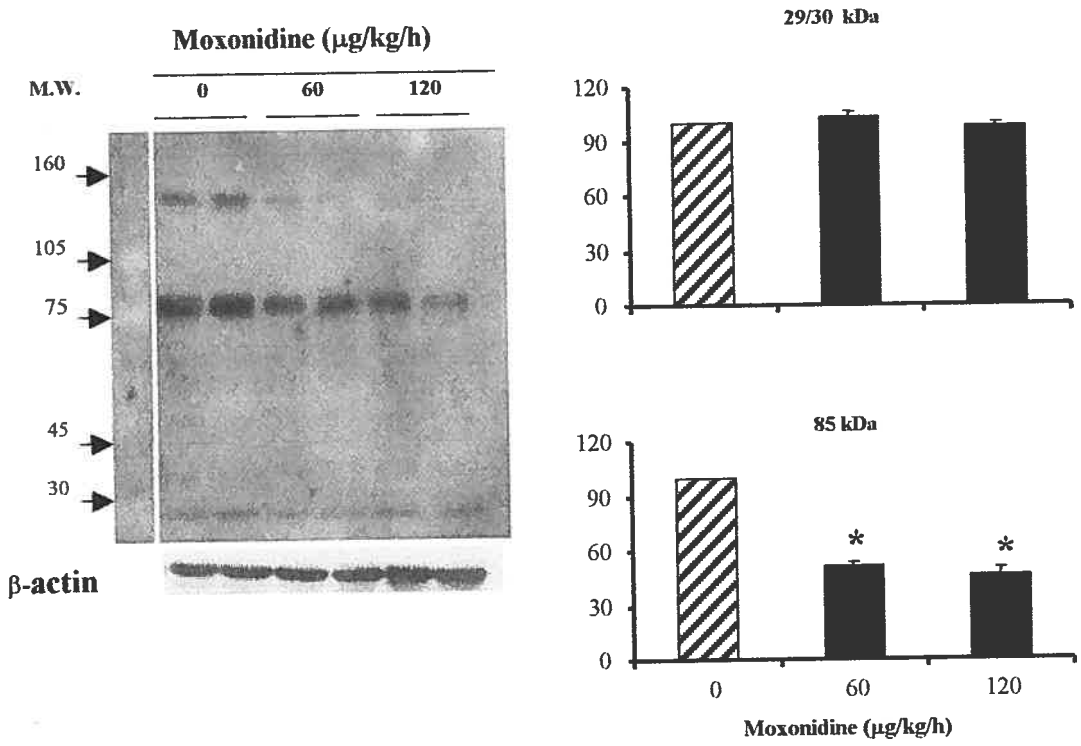
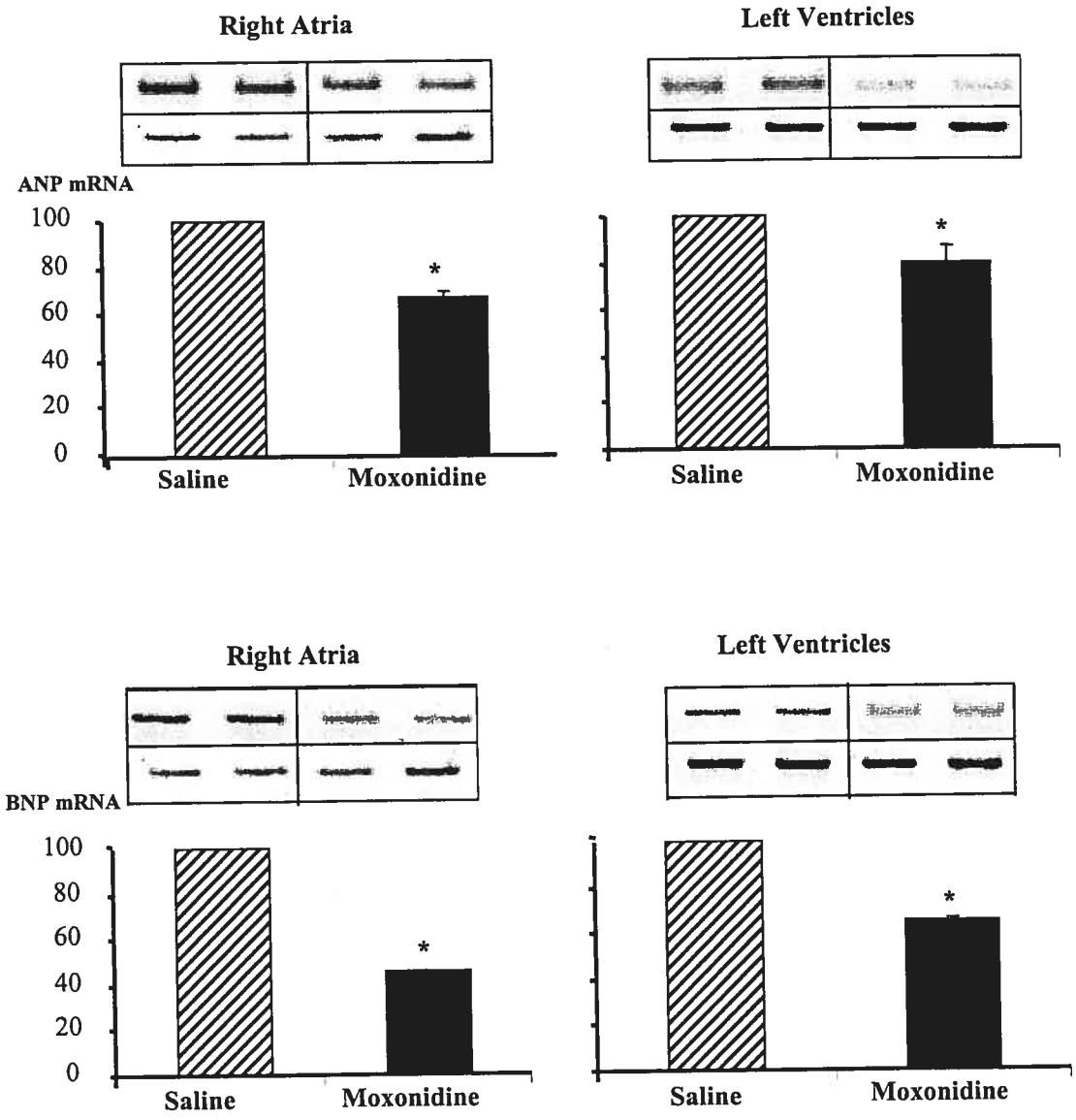

Moxonidine (μg/kg/h)
M.W.
0
60
120
160
105
75
45
30
β-actin
29/30 kDa
120
90
60
30
0
85 kDa
120
90
60
30
0
*
*
0
60
120
Moxonidine (μg/kg/h)

Figure 2


Right Atria
Left Ventricles
ANP mRNA
100
80
60
40
20
0
*
Saline
Moxonidine
Right Atria
Left Ventricles
BNP mRNA
100
80
60
40
20
0
*
Saline
Moxonidine

CHAPITRE 6

Cette étude démontre la présence des récepteurs α_2-adrénergiques dans le cœur. Cette étude permet aussi de distinguer entre les récepteurs I_1 et les α_2-adrénergiques dans le cœur qui était accomplie par différentes approches. Nos travaux ont démontré que les récepteurs aux imidazolines, mais pas les récepteurs α_2-adrénergiques, sont régulés lors des désordres cardiovasculaires et face au traitement chronique in vivo à un agoniste sélectif pour les récepteurs I_1. Cette régulation des récepteurs I_1 en réponse au changement physiologique et pharmacologique, renforce en plus l'identification des récepteurs I_1 dans le cœur et suggère que ces récepteurs jouent un rôle dans les maladies cardiovasculaires.

J Pharmacol Exp Therap 2004

CHAPITRE 6

IMIDAZOLINE RECEPTORS BUT NOT ALPHA2 ADRENOCEPTORS ARE REGULATED IN SHR HEART BY CHRONIC MOXONIDINE TREATMENT

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Running title: Cardiac Imidazoline I_1 receptors in SHR

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E-mail: [redacted]

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6.1-ABSTRACT

We have recently identified imidazoline I_1-receptors in the heart. In the present study, we tested regulation of cardiac I_1-receptors vs. α_2 adrenoceptors in response to hypertension and to chronic exposure to agonist. Spontaneously hypertensive rats (SHR, 12-14 weeks old) received moxonidine (10, 60 and 120 μg/kg/h, s.c.) for 1 and 4 weeks. Autoradiographic binding of ^{125}I-paraiodoclonidine (^{125}I-PIC, 0.5 nM, 1h, 22°C) and inhibition of binding with epinephrine (10^{-10} to 10^{-5} M) demonstrated the presence of α_2-adrenoceptors in heart atria and ventricles. Immunoblotting and RT-PCR identified α_{2A}- α_{2B}- and α_{2C}-adrenoceptor proteins and mRNA, respectively. However, compared to normotensive controls, cardiac α_2-adrenoceptor kinetic parameters, receptor proteins, and mRNAs were not altered in SHR with or without moxonidine treatment. In contrast, autoradiography showed that up-regulated atrial I_1-receptors in SHR are dose-dependently normalized by 1 week, with no additional effect after 4 weeks of treatment. Moxonidine (120 μg/kg/h) decreased Bmax in right (40.0±2.9 to 7.0±0.6 fmol/unit area, $p<0.01$) and left (27.7±2.8 to 7.1±0.4 fmol/unit area, $p<0.01$) atria, and decreased the 85 and 29 kDa imidazoline receptor protein bands, in right atria, to 51.8±3.0% ($p<0.01$) and 82.7±5.2% ($p<0.03$) of vehicle-treated SHR, respectively. Moxonidine-associated percent decrease in Bmax only correlated with the 85 kDa protein ($R^2 = 0.57$; $p<0.006$), suggesting that this protein may represent I_1-receptors. The weak but significant correlation between the two imidazoline receptor proteins ($R^2 = 0.28$; $p<0.03$), implies that they arise from the same gene. In conclusion, the heart possesses I_1-receptors and α_2 adrenoceptors, but only I_1-receptors are responsive to hypertension and to chronic *in vivo* treatment with a selective I_1-receptor agonist.

6.2-INTRODUCTION

Most of the centrally acting antihypertensive drugs, such as clonidine and related imidazoline derivatives mediate sympathoinhibition, not only via activation of central nervous α_2-adrenoceptors, but also via imidazoline I_1-receptors (Bousquet, 1997; Bricca et al. 1989). Imidazoline I_1-receptors are non-adrenergic and non-cholinergic neurotransmitter receptors that possess low affinity for norepinephrine and other catecholamines. I_1-receptors are mainly found in the brainstem, adrenal chromaffin cells and kidneys. In addition, we have recently identified I_1-receptors in heart atria and ventricles, and shown that atrial I_1-receptors are up-regulated in rat hypertension and ventricular I_1-receptors are up-regulated in human and hamster heart failure (El-Ayoubi et al., 2002a). In other studies we demonstrated that acute injections of moxonidine, an imidazoline compound that shows 40 times higher affinity to I_1-receptor vs. α_2-adrenoceptors, are associated with enhanced release of atrial natriuretic peptide (ANP) (Mukaddam-Daher and Gutkowska, 2000), a cardiac hormone involved in pressure and volume homeostasis. Taken together, these studies led us to suggest that heart I_1-receptors are functional and may be involved in cardiovascular regulation.

Previous binding studies reported ^{3}H-idazoxan binding sites (I_2-receptors) but not I_1-receptors in human atrial appendage, but functionally, these receptors were different from presynaptic imidazoline receptors implicated in inhibition of noradrenaline release. Accordingly, atrial presynaptic imidazoline receptors were considered non-I_1 non-I_2 receptors, and the effects of moxonidine to inhibit noradrenaline release in atrial appendages were attributed to presynaptic α_2-adrenoceptors (Molderings et al, 1999). In contrast, consistent with the presence of I_1-receptors in the heart, Schäfer et al (2003) have recently shown in isolated perfused rats hearts that moxonidine is able to decrease noradrenaline release independently of α_2-adrenoceptors.

In fact, functional separation between imidazoline I_1-receptors and α_2-adrenoceptors is rather difficult, because these receptors are often co-localized and ligands with affinity to imidazoline I_1-receptors also bind to α_2-adrenoceptors (Bousquet, 1997).

However, previous studies indicate that imidazoline receptors and α_2 adrenoceptors are subject to pathophysiological and pharmacological regulation (Yakubu et al., 1990; Ivanov et al., 1998; Zhu et al., 1997; Ernsberger et al., 1991). Therefore, the aim of the present studies was to test regulation of cardiac I_1-receptors vs. α_2 adrenoceptors, by showing that I_1-receptors, but not α_2 adrenoceptors are regulated in hypertension and in response to exposure to agonist. Accordingly, studies were performed to: 1) demonstrate the presence of α_2 adrenoceptors in the heart and their possible regulation in hypertension, and 2) to investigate the effect of chronic *in vivo* exposure to moxonidine on I_1-receptors and α_2 adrenoceptors in hearts of normotensive rats and spontaneously hypertensive rats (SHR) with established hypertension.

6.3-METHODS

Female Spontaneously Hypertensive Rats (SHR, 12–14 weeks old) with established hypertension and age-matched normotensive Wistar-Kyoto (WKY) and Sprague Dawley (SD) rats were purchased from Charles River (St. Constant, QC). Animals were housed in temperature and light controlled room with food and water *ad libitum*, and maintained for at least 3 days before experimentation. Experiments were performed following the approval of the Bioethics Committee of CHUM, according to the Canadian Guidelines.

Alzet osmotic minipumps (2ML1 & 2ML4, Alzet Corp.) were implanted subcutaneously in SHR, under isoflurane anesthesia, as we have previously described (Menaouar et al., 2002). These mini-pumps allowed continuous delivery of moxonidine (Generous gift from Solvay Pharmaceuticals, Germany) or saline vehicle at the rate of 10 μL/h (2ML1), for one week, and 2.5 μL/h (2ML4) for 4 weeks. The concentrations of moxonidine were adjusted to allow delivery of 10, 60 & 120 μg/kg/h. The solution of moxonidine was prepared by dissolving the drug in isotonic saline, pH<6.5, then pH adjusted to 7.0-7.4 by NaOH. Rats were sacrificed after 1 and 4 weeks of vehicle and moxonidine treatment, and heart atria and ventricles were separated, snap-frozen in pre-chilled isopentane, then stored at –80°C, for receptor analysis by autoradiographic binding, immunoblotting, and RT-PCR.

To rule out the influence of blood pressure on receptor regulation, another group of SHR was treated with hydralazine, given at 30 mg/kg/day, in drinking water, for 1 week. The effectiveness of hydralazine was verified by tail cuff measurement of systolic blood pressure before and after 1-week treatment. Then, rats were sacrificed and tissues collected as described above.

Autoradiography

Autoradiography of heart I_1-receptors and α_2-adrenoceptors was performed on frozen heart sections from WKY and SD rats, and from saline- and moxonidine-treated SHR, using radiolabelled paraiodoclonidine (^{125}I-PIC; 2200 Ci/mmol; New England Nuclear, Boston, MA) as we have previously described (El-Ayoubi et al., 2002a). Because ^{125}I-PIC binds to both receptor types, autoradiography was performed, separately, in conditions that favor α_2- adrenoceptor binding and in conditions that favor I_1-receptor binding. For α_2-adrenoceptors, the slides were incubated for 1h at 22^{0}C with 0.5 nM ^{125}I-PIC in incubation buffer: in mM 50 Tris-HC (pH 7.7), 5 EDTA, 5 EGTA, 10 $MgCl_2$, and 50 μM phenylmethylsulfonyl fluoride (PMSF). Binding was inhibited by increasing concentrations of epinephrine (10^{-10} to 10^{-5} M). Binding in the presence of 10^{-4} M piperoxan was considered non-specific. After several washes, the slides were dried, exposed in phosphor-sensitive cassette for 48h, then scanned, visualized, and quantified by PhosphorImager (ImageQuant, Molecular Dynamics, Sunnyvale, CA).

Autoradiography for I_1-receptors was performed under identical incubation conditions, except for prior incubation of slides with 1 mM phenoxybenzamine and 0.5 mM ethylmaleimide for 35 min at room temperature, to irreversibly inhibit adrenoceptor binding; and by decreasing the concentration of $MgCl_2$ in the incubation buffer to 0.5 mM, conditions that favor binding to I_1-receptors (Ernsberger et al., 1995). Binding of ^{125}I-PIC was competitively inhibited by increasing concentrations (10^{-12} to 10^{-5} M) of moxonidine

Membrane preparation and Immunoblotting

Membranes of ventricular and atrial tissues were prepared in sucrose buffer as previously described (El-Ayoubi et al, 2002a). Protein content was measured spectrophotometrically, using BSA as standard.

Immunoblotting was performed (El-Ayoubi et al., 2002a) using 30 μg denatured protein samples from cardiac tissues and incubation of blots with rat α_{2A}-, α_{2B}-, α_{2C}-adrenoceptor antiserum (1:500, Santa-Cruz Biotech), or anti-imidazoline receptor antiserum and non-immune antiserum (Generous gift from S. Regunathan, Jackson, MS) diluted 1:1000, or with anti-β-actin (1:500). The blots were incubated with horseradish peroxidase-conjugated anti-rabbit IgG antiserum (1:5000). Immunoreactive bands were visualized by Amersham's enhanced chemiluminescence (ECL) detection system (Amersham ECL hyperfilm), according to the manufacturer's instructions.

Total RNA Extraction and RT-PCR.

Total RNA was extracted from the rat heart tissues using Trizol reagent (Life Technologies, Inc.,) according to the protocol described by the manufacturer. PCR reactions were performed (Zou and Cowley, 2000) using specific primer pairs for rat α_{2A}-, α_{2B}-, α_{2C}- receptors, or β-actin (Operon Technologies, Alameda, CA, USA). After electrophoresis on agarose gel in the presence of ethidium bromide, fluorescent PCR products were scanned, counted and analyzed with the ImageQuant software. These data were normalized to the corresponding values of β-actin PCR product in the same samples.

Data Analysis

The equilibrium dissociation constant (Kd) and maximum binding capacity (Bmax) for the ligands used in autoradiography were calculated by the non-linear method using the Ligand computer program (Elsevier-Biosoft, Cambridge, UK). Densitometric measurements of immunoblots were performed using Scion computer program (NIH, Bethesda, MD). Correlation coefficients were calculated from linear regression (GraphPad Prism; GraphPad Software, Inc., San Diego CA). Differences in data obtained from vehicle- or moxonidine-treated rats were compared by non-paired Student's t-test. $P<0.05$ was considered significant. All data are expressed as mean ± SEM.

6.4-RESULTS

Cardiac α_2-adrenoceptors

Autoradiographic binding of ^{125}I-PIC to heart atrial and ventricular sections was inhibited by increasing concentrations of epinephrine. Kinetic parameters obtained from competitive inhibition curves (Table I) revealed that α_2-adrenoceptor affinity (Kd $\cong$ 2.5 nM) and Bmax in right atria (12.8±0.7 vs. 13.4±0.9 fmol/unit area), left atria (12.8±0.4 vs. 11.7±0.7 fmol/unit area) and left ventricles (11.7±1.1 vs. 12.2±0.5 fmol/unit area) were not altered in SHR as compared to WKY rats. Binding to cardiac α_2-adrenoceptors in SHR was also not altered by chronic *in vivo* moxonidine treatment. Bmax remained in 120 µg/kg/h moxonidine-treated SHR right atria at 11.9±0.9 fmol/unit area and Kd at 2.3±0.3 nM. Similarly, kinetic parameters obtained in left atria and left ventricles were not altered in vehicle- or moxonidine-treated SHR (Table I).

Three α_2-adrenoceptor subtypes were identified in cardiac tissues of SD and SHR, by immunoblotting. Densitometric measurements of the bands corresponding to α_{2A}- (Fig. 1), α_{2B}- and α_{2C}-adrenoceptors (not shown) were not significantly different in right atria and left ventricles of SHR vs. SD, nor in vehicle- and moxonidine-treated SHR, where variation did not exceed 10%. Furthermore, levels of three subtypes of α_2-adrenoceptor mRNA detected in right and left atria and left ventricles of SHR were also not significantly different among vehicle- or moxonidine-treated groups, where variation did not exceed 10% (Fig. 2).

Cardiac Imidazoline Receptors

Autoradiography showed that total specific binding of ^{125}I-PIC to I_1-receptors was higher in SHR atria (162%) as compared to normotensive WKY rats, considered as 100%. Also, total specific binding in atria decreased after treatment with moxonidine at 10, 60 and 120 µg/kg/h for 1 week (Fig. 3).

Competitive inhibition curves were plotted from values obtained from normotensive and hypertensive vehicle- and moxonidine-treated rats and presented as percent B/B_0, where B and B_0 represent respectively, binding with and without moxonidine (Fig. 3). Kinetic parameters calculated from these curves using the Ligand computer program, revealed that 1-week treatment dose-dependently decreased Bmax in SHR right and left atria. At the lowest dose of 10 μg moxonidine, Bmax decreased from 40.0±2.9 to 18.2±0.4 fmol/unit area ($p<0.01$) in right atria, and from 27.7±2.8 to 12.3±0.6 fmol/unit area ($p<0.04$) in left atria. The doses of 60 and 120 μg moxonidine decreased Bmax in rat right and left atria to values not significantly different from 2 normotensive controls (Table II). Four-week treatment did not have additional effects, so that at 120 μg moxonidine, Bmax in right atria represented 9.0±0.3 fmol/unit area. Moxonidine treatment did not affect Bmax and Kd of I_1-receptors in right and left ventricles of moxonidine- and vehicle-treated SHR (Table II).

The presence of three immunoreactive imidazoline receptor protein bands was shown in cardiac tissues by immunoblotting. The apparent molecular masses of these proteins were around 160, 85, and 29/30 kDa. Densitometric measurements of bands corresponding to the 160 kDa band was only slightly increased in atria of SHR, and almost not detected in normotensive SD rats and in moxonidine-treated SHR for 1 or 4 weeks. On the other hand, the density of bands corresponding to 85 kDa protein increased significantly ($p<0.05$) in right atria of SHR to 134.6±3.3% compared to normotensive control (considered as 100%).

Fig. 4 shows that, compared to vehicle-treated SHR (considered as 100%), chronic moxonidine treatment resulted in a significant ($p<0.01$) decrease in the intensity of the bands corresponding to 85 kDa proteins to represent 83.4±1.9%, 59.9±2.7%, and 51.8±3.0% in 10, 60 and 120 μg moxonidine-treated SHR for 1 week, respectively.

Treatment with moxonidine at 60 and 120 μg/kg/h for 4 weeks, resulted in a mild additional decrease in the intensity of the 85 kDa bands to 51.1±2.2% and 46.8±3.3%, respectively (Fig. 4). A modest increase in the intensity of ~29 kDa band (not shown) was observed in right atria of SHR (107±2%) that decreased to 94±1%, 87±3%, and 83±5% after one week of moxonidine at 10, 60 and 120 μg, respectively. In left ventricles, chronic treatment of SHR with moxonidine (120 μg) for 1 and 4 weeks did not alter the intensity of the bands corresponding to 85 kDa, but slightly decreased the 29 kDa band to 89±3%, and after 4 weeks to 89±1%.

Compared to corresponding WKY (considered as 100%), the percent increase in SHR right atrial Bmax correlated with the percent increase in the density of the 85 kDa band ($R^2 = 0.7744$; $p < 0.03$), but not with the 29 kDa protein band. Moxonidine treatment resulted in a dose-dependent decrease in Bmax and in the 85 kDa band as compared to corresponding saline-vehicle treated SHR (considered as 100%). Fig. 5 shows that the percent decrease in Bmax correlated with the percent decrease in the 85kDa band ($R^2 = 0.5700$; $p < 0.006$), but not with the 29 kDa band ($R^2 = 0.1754$; N.S.), suggesting that the 85 kDa protein may represent imidazoline I_1-receptors in the heart. A weak, but significant correlation was found between the 85 kDa and the 29 kDa protein band ($R^2 = 0.2717$; $p < 0.03$), in moxonidine-treated SHR, implying that the two receptor proteins arise from the same gene.

Treatment of SHR with hydralazine for 1 week, resulted in blood pressure reduction from 193±8 to 135±5 mmHg, $p<0.02$, whereas blood pressure remained in control rats at 186±11 mmHg. However, hydralazine treatment did not alter imidazoline receptor proteins measured by immunoblotting.

6.5-DISCUSSION

The major findings of this study are: 1) First time localization of α_2-adrenoceptors in heart atria and ventricles; and 2) demonstration that heart imidazoline I_1-receptors but not α_2-adrenoceptors are regulated in SHR, and in response to chronic *in vivo* exposure to a selective imidazoline receptor agonist, suggesting that heart I_1-receptors are subject to regulation. In addition, 3) the parallel change in receptor Bmax and the 85 kDa imidazoline receptor protein, suggest that this protein may represent cardiac I_1-receptors.

Pharmacologic and molecular cloning studies have revealed three α_2-adrenoceptor subtypes: α_{2A} (α_{2D} in rats), α_{2B}, and α_{2C} (Link et al., 1996; Altman et al., 1999). In the human heart, mRNA for all 3 α_2-adrenoceptors subtypes have been detected by PCR (Brodde and Michel, 1999). However, probably due to very low expression relative to α_1- and β_1-adrenoceptors, previous studies have not been successful in demonstrating α_2-adrenoceptors in the heart at the protein level through radioligand binding studies (Brodde and Michel, 1999). In the present study, demonstration of α_2-adrenoceptors in the heart was achieved by multiple approaches. Quantitative receptor autoradiography was used in conditions where binding of ^{125}I-PIC to adrenoceptors vs. I_1-receptors was optimized by using high $MgCl_2$ (10 mM) concentration in the incubation buffer (Ernsberger et al., 1995). Furthermore, because radioligands cannot fully discriminate between α_2-adrenoceptor subtypes, further identification was obtained by immunoblots and RT-PCR, using specific rabbit polyclonal antibodies and primers for each subtype (Zou and Cowley, 2000). However, cellular localization of these receptors needs further experiments, since receptor subtypes were detected in whole cardiac tissue which involves several cell types, including fibroblasts and myocytes, myocardial blood vessels, nerve terminals and intracardiac neurons (Armour, 1999).

The α_{2A}, abundant in the CNS, mainly in brain stem, is directly involved in regulating sympathetic outflow, appears to be the major presynaptic autoinhibitory receptor subtype (Altman et al, 1999). The α_{2B} is more abundant in arterial vascular smooth muscle cells and mostly responsible for vasoconstriction, and is responsive to altered salt handling. The function of α_{2C} is not yet clear, but it may be the presynaptic autoreceptor in human atria (Rump et al., 1995; Hein, 2001).

The physiological significance of α_2-adrenoceptors in various heart chambers is beyond the scope of the present study. This study, however, provides strong evidence that α_2-adrenoceptors are present, albeit at low levels, in the rat heart atria and ventricles, at the levels of synthesis, protein expression and binding activity, but these receptors appear not to be regulated by moxonidine, a selective agonist of imidazoline I_1-receptors. Since brain α_2-adrenoceptors have been shown to be selectively down-regulated in response to α_2-adrenoceptor agonists (Yakubu et al., 1990), and kidney imidazoline receptors to be down-regulated in response to imidazoline receptor agonists (Hamilton et al., 1993), the present findings imply that α_2-adrenoceptors in the heart interact weakly with moxonidine.

Most importantly, the present study confirms our previous finding that I_1-receptors are present in the heart, and extend to demonstrate that up-regulated atrial I_1-receptors in SHR (El-Ayoubi et al., 2002a) are normalized by chronic *in vivo* exposure to I_1-receptor agonist. These receptors appear to be unrelated to imidazoline I_2-receptors, previously identified in the heart (Molderings and Gothert, 1999), because, by definition, the ligands used in the present study (^{125}I-PIC and moxonidine) show very low affinity to I_2-receptors (Bousquet, 1997).Immunoblotting of heart membranes showed multiple molecular mass imidazoline receptor proteins similar to those so far described in brain and heart

(El-Ayoubi et al., 2002a). Levels of 85 kDa proteins were increased in atria of untreated SHR as compared to normotensive rats.

Chronic moxonidine treatment, for short and long duration, was associated with decreased density of the 85 kDa bands in SHR atria. It is interesting to note that atrial 85 kDa but not the 29 kDa imidazoline receptor proteins vary in parallel to values of Bmax for I_1-sites determined by ^{125}I-PIC binding. This correlation leads us to propose that the 85 kDa protein may represent I_1-receptors in the heart, as has been suggested by Ivanov et al. (1998). Furthermore, the positive correlation between changes in the two receptor proteins, implies that they may arise from the same gene.Regulation of imidazoline receptors has been previously reported in other tissues and under different physiological and pharmacological manipulations, usually in a manner distinct from α_2-adrenoceptors. Chronic treatment with the prototypic antidepressant imipramine, down-regulates I_1-receptors in rat brainstem, without affecting α_2-adrenoceptors (Zhu et al, 1997). Also, renal I_1-receptors are up-regulated by subpressor doses of angiotensin II infusion (Ernsberger et al., 1991), and in kidneys of SHR (El-Ayoubi et al., 2002b), while α_2-adrenoceptors are either unchanged or decreased in 1K1C rat kidneys (Li et al., 1994).

In the present study, imidazoline receptors were not different in hearts of 2 normotensive strains, WKY and SD, but up-regulated in SHR hearts, then normalized and down-regulated after rat treatment with moxonidine, for short or long duration. The mechanisms involved in receptor up-regulation may include sympathetic over-activity, elevated blood pressure, increased cardiac mass and activated intracardiac neurohormones, such as angiotensin II and norepinephrine. Treatment with moxonidine inhibits or counteracts these effects, and eventually may indirectly lead to down-regulation of its receptor. We have previously shown that moxonidine dose-dependently reduced blood pressure in SHR (Menaouar et al., 2002). However, the dose of 10 μg moxonidine, which had no effect on blood

pressure in those rats (Menaouar et al., 2002), significantly reduced I_1-receptor protein and Bmax.

Furthermore, treatment of SHR with hydralazine, a vasodilator antihypertensive compound that reduced blood pressure to a similar magnitude achieved by 120 μg moxonidine, had no effect on imidazoline receptor proteins. Further studies are needed to clarify the mechanisms involved, but the present results argue against receptor down-regulation occurring in response to reduction in blood pressure per se, and in favor of a direct effect of the ligand on the receptor. Other investigators demonstrated that stimulation of I_1-receptor with moxonidine leads to activation of PC-PLC and generation of DAG, which activates several isoforms of protein kinase C (PKC) (Ernsberger, 1999). PKC results in functional desensitization of the I_1-receptor through phosphorylation of serine and threonine residues in the receptor intracellular loop (Eason and Ligett, 1996).

In conclusion, this study demonstrates that heart I_1-receptors but not α_2-adrenoceptors are up-regulated in SHR and normalized by chronic antihypertensive treatment with moxonidine. Cardiac I_1-receptor normalization occurred after 1-week of treatment, the time point when moxonidine resulted in reversal of left ventricular hypertrophy in these rats (Menaouar et al., 2002). Also, the presence of I_1-receptors in atria, tissues known to secrete or respond to natriuretic peptides. ANP and BNP, suggest a functional relationship between the two systems. Therefore, heart I_1-receptors are subject to regulation by the cardiovascular environment. Future antihypertensive treatment with imidazoline drugs should consider the heart as a major target organ.

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6.7-FIGURE LEGENDS

Fig. 1: Representative immunoblot of α_2-adrenoceptor subtypes (α_{2A}, α_{2B}, α_{2C}) and β-actin, and densitometric data of α_{2A} measured in right atria and left ventricles of SD and SHR after treatment with moxonidine (0, 60 & 120 μg/kg/h) for 1 and 4 weeks. Data normalized to corresponding β-actin are presented as percent change from SD and vehicle-treated SHR.

Fig. 2: RT-PCR mRNA products of α_2-adrenoeceptors and β-actin in right and left atria and left ventricles of SHR treated with moxonidine (0, 60 & 120 μg/kg/h) for 4 weeks.

Fig. 3: Top: Representative autoradiography of total ^{125}I-PIC binding to right atrial tissue sections (after irreversible inhibition of α-adrenoeceptor binding) in WKY and SHR after 1-week treatment with moxonidine (0, 10, 60, 120 μg/kg/h).

Bottom: Specific binding of ^{125}I-PIC to right atrial sections in WKY and SHR after 1-week treatment with moxonidine (0, 10, 60, 120 μg/kg/h). Data are presented as $\%B/B_0$, where B and B_0 represent binding in the absence and presence of increasing concentrations (10^{-12} to 10^{-5} M) of inhibiting ligand.

Fig. 4: Representative immunoblot and densitometric measurement of the 85 kDa imidazoline receptor protein in right atria of SHR after treatment with moxonidine (0, 10, 60 & 120 μg/kg/h) for 1 week and 60 & 120 μg/kg/h for 4 weeks. Data normalized to corresponding β-actin are presented as percent change from vehicle-treated SHR (considered as 100%). *$p<0.01$ vs. vehicle-treated SHR.

Fig. 5: Top: Correlation between percent decrease in moxonidine-treated SHR (vehicle-treated SHR considered as 100%) right atrial Bmax obtained from competitive binding assays vs. percent decrease in the density of the 29 kDa (dotted line) and 85 kDa (solid line) bands obtained by immunoblotting.Bottom: Correlation between percent change in the density of right atrial 85 kDa vs. 29 kDa bands obtained by immunoblotting.

Figure 1

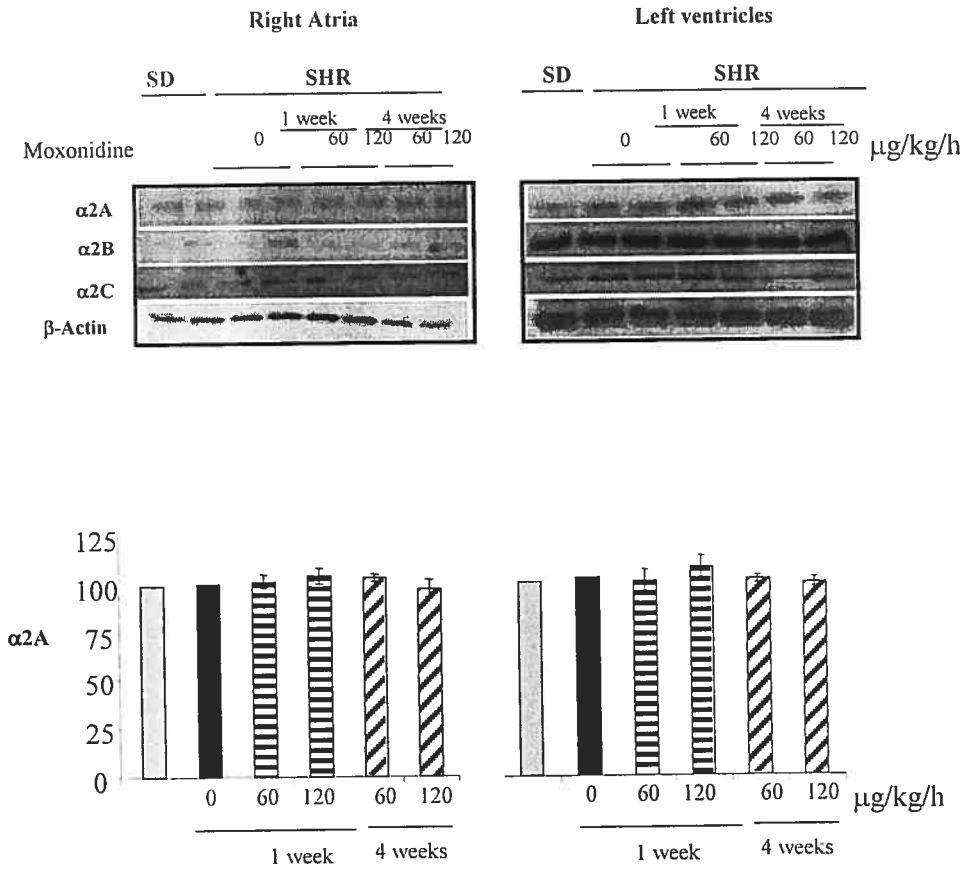

Right Atria
Left ventricles
SD
SHR
SD
SHR
1 week
4 weeks
1 week
4 weeks
Moxonidine
0
60
120
60
120
0
60
120
60
120
μg/kg/h
α2A
α2B
α2C
β-Actin
α2A
125
100
75
50
25
0
0
60
120
60
120
0
60
120
60
120
μg/kg/h
1 week
4 weeks
1 week
4 weeks

Figure 2

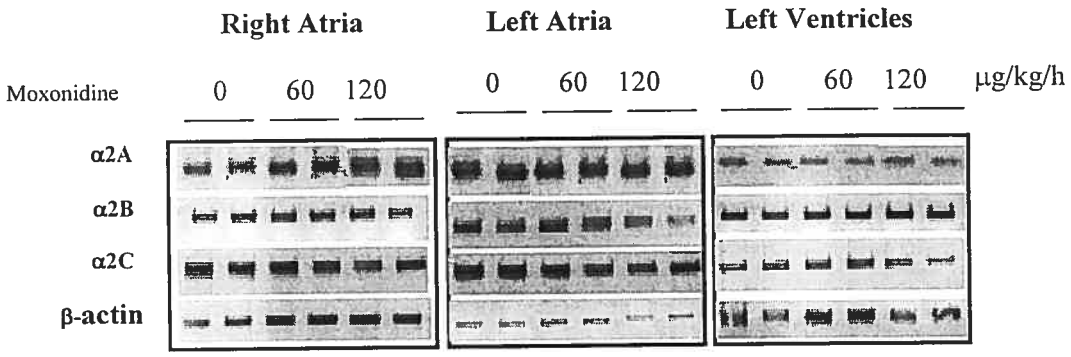

Right Atria
Left Atria
Left Ventricles
Moxonidine
0
60
120
0
60
120
0
60
120
μg/kg/h
α2A
α2B
α2C
β-actin

Figure 3

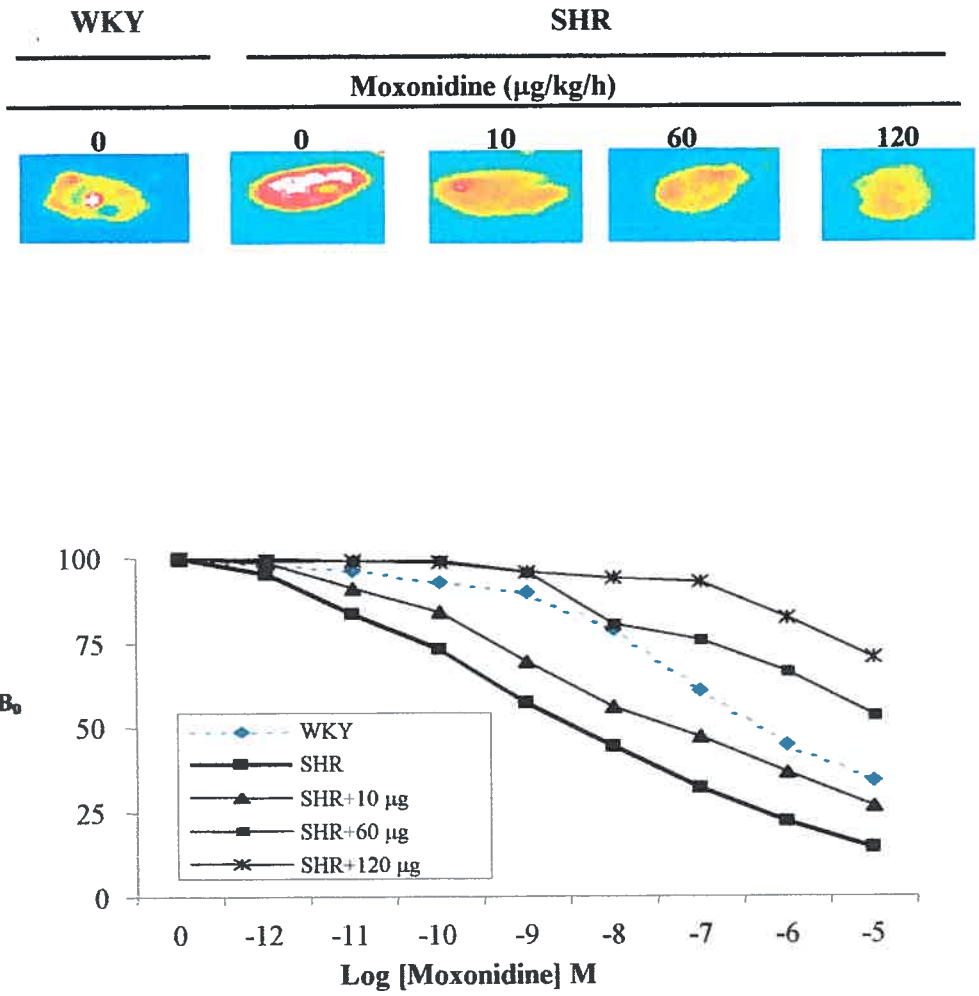

WKY
SHR
Moxonidine (µg/kg/h)
0
0
10
60
120
100
75
50
25
0
% B/B0
WKY
SHR
SHR+10 µg
SHR+60 µg
SHR+120 µg
0
-12
-11
-10
-9
-8
-7
-6
-5
Log [Moxonidine] M

Figure 4

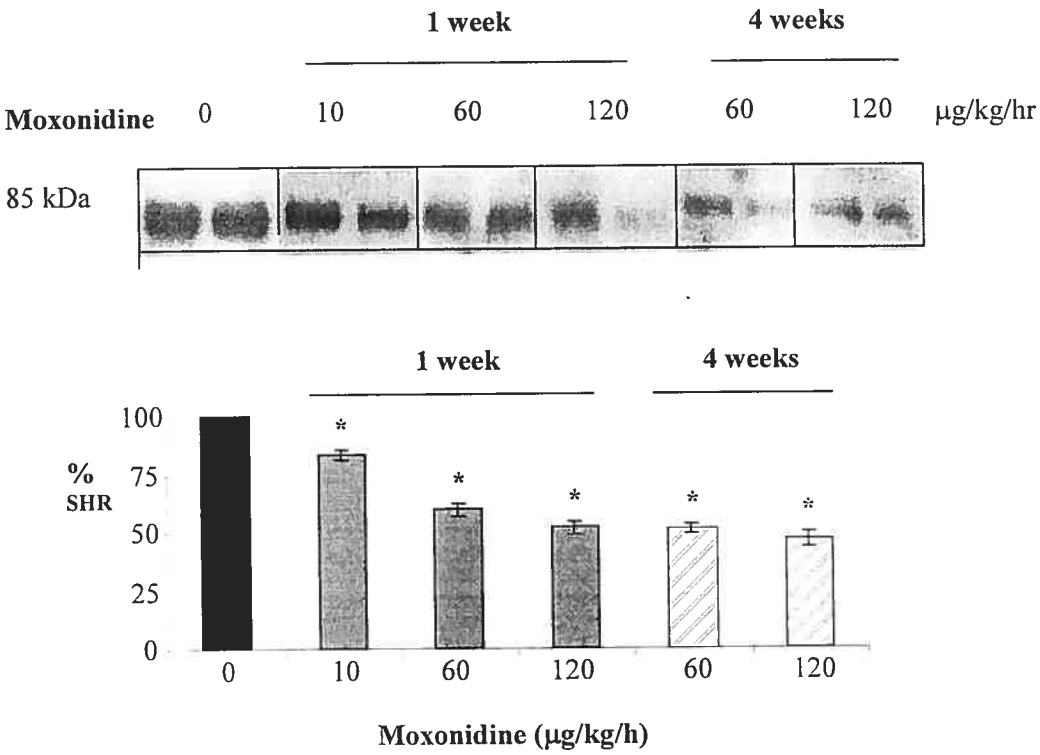
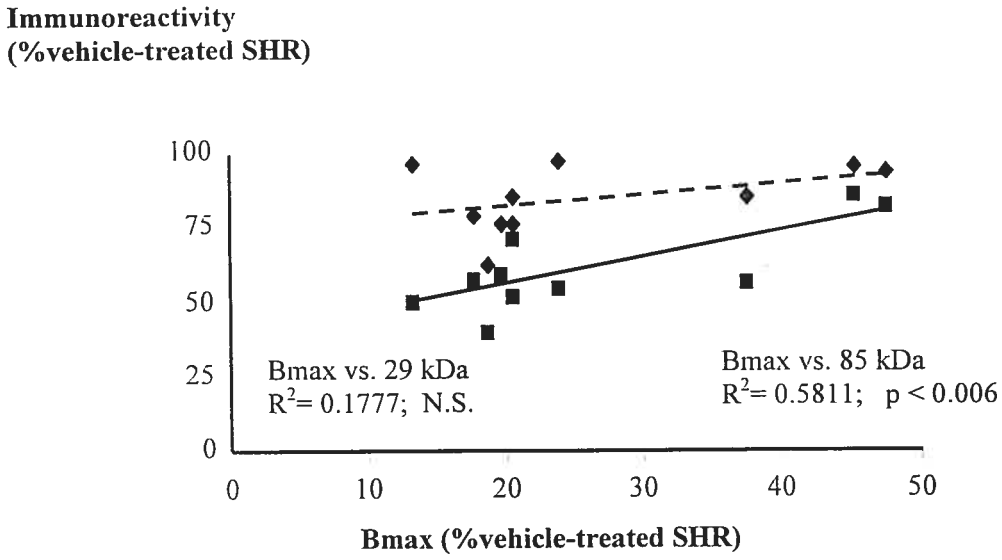
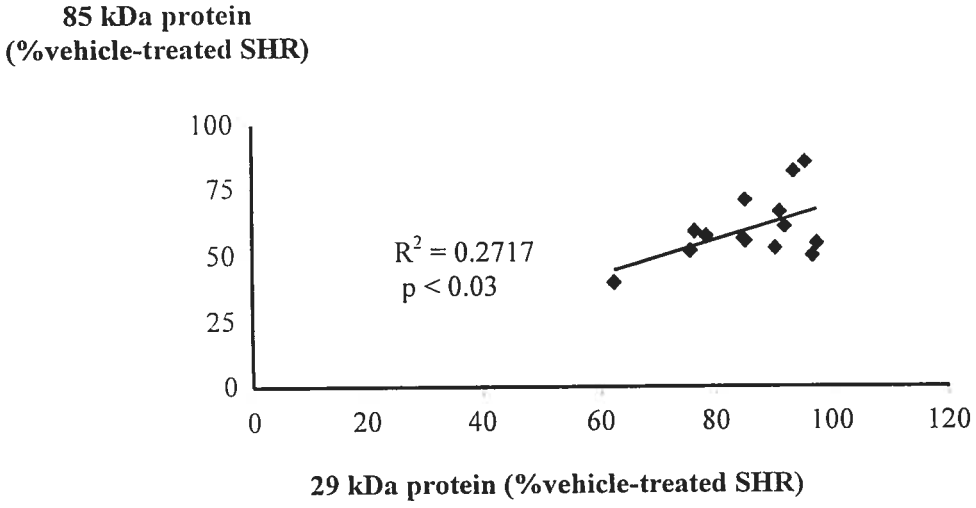

1 week
4 weeks
Moxonidine
0
10
60
120
60
120
µg/kg/hr
85 kDa
1 week
4 weeks
% SHR
100
75
50
25
0
*
0
10
60
120
60
120
Moxonidine (µg/kg/h)

Figure 5


Immunoreactivity
(%vehicle-treated SHR)
100
75
50
25
0
0
10
20
30
40
50
Bmax vs. 29 kDa
R2= 0.1777; N.S.
Bmax vs. 85 kDa
R2= 0.5811; p < 0.006
Bmax (%vehicle-treated SHR)


85 kDa protein
(%vehicle-treated SHR)
100
75
50
25
0
0
20
40
60
80
100
120
R2 = 0.2717
p < 0.03
29 kDa protein (%vehicle-treated SHR)

CHAPITRE 7

DISCUSSION

L'ensemble de ces études démontrent que :1) le traitement chronique des SHR à la moxonidine diminue la pression artérielle, régresse l'hypertrophie ventriculaire gauche et ces effets sont associés à une augmentation de l'expression cardiaque et de niveau plasmatique de l'ANP durant une semaine de traitement et la normalisation de l'expression cardiaque et du niveau plasmatique d'ANP pendant un mois de traitement chronique à la moxonidine; 2) l'administration intraveineuse (i.v.) de la moxonidine, chez les rats normotendus (SD et WKY) et les rats SHR, stimule la diurèse, la natriurèse, la kaliurèse, augmente l'excrétion de GMPc, sans que ces réponses rénales ne soient influencées par l'hypertension et que ces réponses rénales sont associées à l'augmentation plasmatique de l'ANP et sont inhibées de façon dose-dépendante par l'anantin, indiquant l'implication de l'ANP dans les effets aigus rénaux de la moxonidine (i.v.); 3) les récepteurs aux imidazolines et α_2-adrénergiques sont identifiés dans les oreillettes et les ventricules de cœur, de même que dans les myocytes et les fibroblastes cardiaques; 4) la densité des récepteurs I_1, mais pas celle des récepteurs α_2-adrénergiques, était augmentée dans les oreillettes des rats hypertendus et dans les ventricules des hamsters cardiomyopathiques et des sujets atteints d'insuffisance cardiaque; 5) la densité des récepteurs I_1 était normalisée suite à l'exposition chronique à la moxonidine *in vivo*, indiquant que les récepteurs I_1 cardiaques répondent au changement physiologique et pharmacologique; 6) les récepteurs I_1 et α_2-adrénergiques cardiaques sont fonctionnels, vu que leur activation *in vitro* par la moxonidine provoque la libération de l'ANP, sans l'intervention centrale.

7.1-Identification des récepteurs I_1 et α_2-adrénergiques dans le cœur

Ces études démontrent pour la première fois la présence des récepteurs I_1 et α_2-adrénergiques dans le cœur.

Les récepteurs I_1 sont identifiés dans les oreillettes et les ventricules cardiaques (El-Ayoubi et coll., 2002). En raison du manque de ligand spécifique pour les récepteurs I_1, diverses techniques ont été utilisées, comme le transfert Western et l'immunohistochimie, en utilisant un anticorps spécifique qui reconnaît les récepteurs I_1 et I_2 (Regunathan et coll., 1996), mais pas les récepteurs α_2-adrénergiques. Puisque l'anticorps ne distingue pas entre les récepteurs I_1 et I_2, d'autres techniques ont été utilisées pour identifier les récepteurs I_1, comme la liaison de l'analogue radiomarqué de la clonidine (^{125}I-PIC) sur les sections gelées et les membranes cardiaques, qui lie les récepteurs I_1 et les récepteurs α_2-adrénergiques, mais pas les récepteurs I_2 (Ernsberger et coll., 1995). Pour mieux caractériser les récepteurs I_1, la liaison membranaire était performée après le blocage irréversible des récepteurs α_2-adrénergiques.

Les récepteurs I_1 étaient identifiés dans le tissu cardiaque entier, qui inclut les myocytes, les fibroblastes, les vaisseaux sanguins, les terminaisons nerveuses, les neurones intracardiaques. Pour spécifier leur localisation, des études préliminaires ont été réalisées sur les myocytes et les fibroblastes cardiaques en culture et les récepteurs I_1 se sont révélés présents dans les myocytes et les fibroblastes cardiaques. Cette identification des récepteurs I_1 dans les myocytes, dépourvus des neurones, affirment que ces récepteurs ne sont pas le type présynaptique déjà présent dans les tissus cardiovasculaires (Moldering et Gothert, 1999; Fuder et Schwarz, 1993).

Nous avons identifié aussi les récepteurs α_2-adrénergiques dans le cœur, Auparavant, les études n'avaient pas réussi à révéler les protéines des récepteurs α_2-adrénergiques dans le cœur (Brodde et Michel, 1999).

Cependant, Eason et Liggett (1993) ont révélé, par RT-PCR, l'expression de chacun des trois sous-types (α_{2A}, α_{2B}, α_{2C}) au niveau du cœur (l'épicarde et l'endocarde). Dans notre étude, on a réussi, par l'utilisation de plusieurs techniques, à identifier les récepteurs α_2-adrénergiques au niveau des protéines dans le cœur. L'autoradiographie quantitative, sur les sections des cœurs et les membranes cardiaques, a été réalisée dans des conditions qui favorisent la liaison de ^{125}I-PIC aux récepteurs adrénergiques (par exemple, la haute concentration de $MgCl_2$ (10 mM) (Ernsberger et coll., 1995). En raison du manque d'agent pharmacologique sélectif qui permette la distinction entre les trois sous-types des α_2-adrénergiques, leur caractérisation dans le cœur a été réalisée par transfert Western, en utilisant un anticorps spécifique pour chacun de sous-type. L'analyse par transfert Western sur les membranes des oreillettes et des ventricules, a mis en évidence les trois sous-types dans le cœur. En plus, la synthèse de chaque sous-type a été détectée par RT-PCR à l'aide des amorces spécifiques, selon Zou et Cowley (2000), donc nos résultats sont en accord avec l'étude d'Eason et Liggett (1993). Cette étude fournit une forte évidence de la présence des récepteurs α_2-adrénergiques dans les oreillettes et les ventricules, au niveau de la synthèse et de l'expression de protéines.

7.2-Régulation des récepteurs I_1 et α_2-adrénergiques cardiaques

Durant ces dernières années, il était très difficile de distinguer le rôle des récepteurs I_1 et des récepteurs α_2-adrénergiques dans l'effet hypotenseur des composés imidazoliniques dû à l'absence des agonistes et des antagonistes spécifiques pour les récepteurs I_1 et du fait que les deux récepteurs sont colocalisés dans la plupart des tissus. C'est pourquoi la signification des récepteurs I_1 était discutable et générait beaucoup de débats sur la présence et le rôle de ces récepteurs. En plus, la moxonidine et la rilmenidine n'ont pas réussi à diminuer la pression sanguine chez les souris mutantes (D79N) où les récepteurs α_{2A}-adrénergiques ont été rendus non fonctionnels suite à une mutation du résidu

d'aspartate à l'asparagine à la position 79 (Asp79Asn), par comparaison aux souris sauvages (WT) (MacMillan et coll., 1998). Cette étude a généré des controverses concernant la présence des récepteurs I_1 et mène à la conclusion que la moxonidine agit sur les récepteurs α_2-adrénergiques pour réduire la pression artérielle. Cependant, des études récentes par Bruban et coll. (2002) ont démontré que l'administration de LNP 509, [cis-/trans-dicyclopropylmethyl-(4,5-diméthyle-4,5-dihydro-3H-pyrrol-2-yl)-amine], agoniste qui agit exclusivement sur les récepteurs aux imidazolines I_1, dans le 4ième ventricule des souris WT et des souris D79N, diminue la pression artérielle. Cet effet hypotenseur était potentialisé chez les rats WT et pas chez les souris D79N, suite à l'administration de l'α-méthyle noradrénaline (agoniste sélectif des récepteurs α_2-adrénergiques) après l'injection de LNP 509. Cette synergie de l'effet entre le LNP 509 et l'α-méthyle noradrénaline chez les souris (WT) et non chez les souris D79N (Bruban et coll., 2002), indique que les récepteurs I_1 et les récepteurs α_{2A}-adrénergiques interagissent synergiquement pour contrôler la pression artérielle (Bruban et coll., 2002). Bousquet et coll. ont observé aussi une synergie entre les récepteurs I_1 et α_{2A}-adrénergiques. Ils ont rapporté que l'administration de S3515, agoniste hautement sélectif pour les récepteurs aux imidazolines, directement dans le système nerveux central, provoque une diminution de la pression sanguine et que cet effet était plus prononcé suite à l'injection de l'α-méthyle noradrénaline (10 min.) après l'administration de S3515 (Bousquet et coll., 2003).

Dans notre étude, la distinction entre les récepteurs I_1 et les α_2-adrénergiques dans le cœur, a été accomplie par différentes approches : nos travaux ont démontré que la régulation *in vivo* des récepteurs aux imidazolines I_1, mais pas les récepteurs α_2-adrénergiques ; 1) en réponse aux désordres cardiovasculaires et 2) face à l'exposition chronique à un agoniste sélectif pour les récepteurs I_1. Par comparaison à l'espèce normale, les récepteurs I_1 ont été surexprimés 2 fois plus, dans les oreillettes des SHR, dans les ventricules des hamsters cardiomyopathiques et dans les ventricules défaillants humains (El-Ayoubi et coll., 2002).

Il n'est pas encore connu si l'augmentation de la densité des récepteurs I_1 est une cause ou un effet de l'élévation de la pression artérielle. L'augmentation de la densité des récepteurs I_1 peut être due à une hausse de la pression artérielle, de l'activité sympathique ou de certains facteurs hormonaux, comme l'angiotensine II et la norépinéphrine. Le groupe de Piletz (Ivanov et al, 1998) avait montré que la stimulation des cellules MEG-01 par la norépinéphrine (10 μM) augmente la densité des récepteurs I_1 et cette augmentation de la densité par la norépinéphrine peut être médié indirectement par les récepteurs α_2-adrénergiques. Vu que lors de l'hypertension le niveau de la norépinéphrine est augmenté, ceci pourrait expliquer en partie l'augmentation des récepteurs I_1 chez les rats hypertendus. D'autre part, l'augmentation de la densité des récepteurs aux imidazolines peut aussi être expliquée par une diminution des ligands endogènes chez les rats SHR. Cependant tous ces mécanismes restent à être explorés.

La densité des récepteurs I_1 auriculaires a été normalisée durant une semaine de traitement chronique par la moxonidine et cette diminution a été maintenue pendant un mois de traitement (El-Ayoubi et coll., 2003). Cependant, le traitement chronique, soit pour une ou 4 semaines, n'avait aucun effet sur les récepteurs α_2-adrénergiques (El-Ayoubi et coll.,2004). Il est important de noter que cette diminution de la densité des récepteurs I_1 après le traitement chronique à la moxonidine n'était pas un mécanisme adaptatif en réponse à la diminution de la pression artérielle, puisque le traitement chronique à l'hydralazine, un vasodilatateur périphérique, diminue la pression sanguine à un niveau comparable à celui de la moxonidine, mais sans affecter la densité des récepteurs aux imidazolines (El-Ayoubi et coll, 2004).

Cette régulation des récepteurs I_1 en réponse au changement physiologique et pharmacologique, renforce en plus l'identification des récepteurs I_1 dans le cœur et suggère que ces récepteurs jouent un rôle dans les maladies cardiovasculaires. Le fait que les récepteurs α_2-adrénergiques cardiaques ne sont pas altérés n'élimine pas le

fait qu'ils sont présents dans le cœur et que leur intégrité est peut-être très nécessaire au fonctionnement des récepteurs I_1.

Cette étude et d'autres études précédentes ont démontré que les récepteurs I_1 et les récepteurs α_2-adrénergiques sont altérés sous plusieurs conditions physiologiques et pharmacologiques. Chez les patients déprimés, les récepteurs I_1 sont surexprimés dans les plaquettes sanguines, qui reflètent les récepteurs I_1 centraux (Piltez et coll., 1996a). La densité des récepteurs α_2-adrénergiques est augmentée dans les cerveaux des sujets victimes du suicide et de la dépression (Meana et coll., 1992). Chez les rats hypertendus, la densité des récepteurs α_2-adrénergiques était réduite dans le cortex cérébral, l'hypothalamus et la medulla oblongata (Olmos et coll., 1991). Pendant l'hypertension, les récepteurs α_2-adrénergiques rénaux ne sont pas changés ou diminués chez les rats "one kidney one clip" (1K1C) (Li et Smyth, 1993). La densité des récepteurs I_1 diminue aussi après le traitement chronique à des agonistes. Leur densité diminue aussi après le traitement des patients déprimés avec les agents antidépresseurs dans les plaquettes sanguins (Piletz et coll., 1996b; Zhu et coll., 1999) et dans le cortex cérébral (Zhu et coll., 1997).

Dans nos études, nous avons utilisé les rats SHR, le modèle le plus souvent étudié d'hypertension humaine primaire (Okamoto et Aoki, 1963). Comme chez l'humaine, l'hypertension chez les SHR est associée à une hyperactivité sélective du tonus sympathique sur le cœur et sur les reins, ces organes qui jouent un rôle capital dans la survenue de l'hypertension artérielle. Les nouveau-nés SHR ont une tension artérielle normale qui commence à augmenter entre la 3^e et la 10^e semaine de vie, et cette tension artérielle augmente rapidement de 30 % au-dessus de cela de WKY (Clubb et coll., 1987; Alemayehu et coll., 2002). À l'âge de 12 semaines, l'hypertension est déjà établie chez ces rats, c'est pourquoi nos études ont été réalisées en utilisant des SHR à 12-14 semaines d'âge. En outre, comme dans l'hypertension humaine, l'augmentation de l'activité sympathique rénale chez les SHR est un dispositif important.

Il était aussi important d'utiliser deux souches de contrôles normotendus, Wistar-Kyoto (WKY, l'origine de SHR) et les rats Sprague-Dawley pour étudier l'effet de l'augmentation de la pression artérielle sans l'influence du "background" génétique. En outre, il y a plusieurs études qui ont démontré que WKY n'est pas le contrôle idéal pour SHR, car ils ont également trouvé une élévation de la pression artérielle chez les jeunes WKY (4 à 5 semaines de vie); ceci peut refléter une différence entre cette souche et d'autres souches normotendues (Lindpaintner et coll., 1992; Stier, 2002). Le groupe d'Aiello et coll. (2004) a rapporté que le WKY développe une hypertrophie au niveau du myocarde indépendante de la surchage pressive, une fibrose et une dysfonction diastolique de façon pas très différente des rats SHR. Cependant, ces paramètres n'étaient pas observés chez les rats Wistar des contrôles normotendus (Aiello et coll., 2004). Ces commentaires ci-dessus ont occasionné des doutes sur le fait de considérer WKY comme un modèle de contrôle génétique approprié pour le SHR.

Ensuite, la caractérisation fonctionnelle des récepteurs I_1 a été déterminée par l'utilisation de la moxonidine, un agoniste sélectif des récepteurs I_1. La moxonidine est un agent antihypertensif efficace d'action centrale utilisé chez le rat et l'humain. Une dose quotidienne de 0,2-0,6 mg, chez les patients présentant une hypertension essentielle faible à modérée, induit une réduction satisfaisante (~30-40 mm Hg) de la pression artérielle durant 24 heures. De plus, environ 90 % d'une dose orale de la moxonidine est absorbée. Les taux plasmatiques maximums ont atteint 30 à 180 min. après la prise orale. La demi-vie moyenne d'élimination plasmatique est de 2,2-2,3 heures (Prichard et coll., 1997).

Pour éviter la variation de la disponibilité et des niveaux plasmatiques de la moxonidine chez les rats, nous avons implanté chez les rats des minipompes osmotiques (minipompes Alzet) en sous-cutané pour diffuser la moxonidine (0, 10, 60 et 120 µg/kg/h) à un débit constant pendant quatre semaines sans interruption. Ces doses de moxonidine sont administrées avec le même écart que celles utilisées par d'autres groupes chez les rats (Ernsberger et coll., 1994;

Ernsberger et Haxhiu, 1997), mais à des doses plus élevées que celles utilisées chez l'homme.

L'utilisation de la moxonidine à des doses élevées dans notre étude est basée sur des études antérieures par Ernsberger et coll. (1997), qui a signalé que la moxonidine traverse plus facilement la barrière hémato-céphalique chez l'homme que chez le rat en raison de la différence de la structure de cette barrière (Ernsberger et coll., 1994), ainsi que le métabolisme et la disposition de la moxonidine chez l'homme sont différents de celui de rats (He et coll., 2000). La moxonidine est très intensivement métabolisée chez les rats; conformément à ceci, un total de sept métabolites ont été détectés chez l'homme tandis qu'un total de quinze métabolites ont été identifiés chez les rats.

La demi-vie de la moxonidine (0,3 mg/kg) est de 0,9 et 1,1 h chez les rats après l'administration intraveineuse ou orale, respectivement (He et coll., 2000). Enfin, la disponibilité biologique déterminée chez l'homme (88 %) (Schachter, 1999) est plus élevée que chez les rats (5 %) (He et coll., 2000).

Nos études *in vivo* incluent l'activation aiguë et chronique des récepteurs I_1 par la moxonidine, qui aboutit à la diminution de la pression artérielle. La moxonidine entraîne une baisse de la pression sanguine par activation sélective des récepteurs I_1 centraux et une inhibition de l'activité du SNS (Ernsberger et coll., 1994; Wenzel et coll., 2000) avec la moindre contribution des récepteurs α_2-adrénergiques centraux. L'inhibition de l'activité de nerf sympathique rénal mène à la diurèse et à la natriurèse, en modulant l'hémodynamie rénale, la libération de rénine et la réabsorption de sodium (DiBona, 2002; Nielsen et coll., 2002; Zou et Cowley, 2000; Junaid et coll., 1999). En plus, la moxonidine peut agir directement sur des récepteurs aux imidazolines et/ou des récepteurs α_2-adrénergiques dans le cortex et la medulla externe de rein (Allan et coll., 1993; Bohmann et coll., 1994; Greven et von Bronewski-Schwarzer, 2001; Bidet et coll., 1990; Limon et coll., 1992) pour stimuler

l'excrétion de sodium. Tous ces mécanismes contribuent à la régulation à court et à long terme de la pression artérielle.

Lors du traitement aigu, l'injection intraveineuse de la moxonidine chez les rats normotendus et hypertendus conscients stimule la diurèse, la natriurèse, l'excrétion de GMPc et l'augmentation d'ANP plasmatique. L'administration intraveineuse de la moxonidine chez les rats conduit à une diminution de la pression artérielle des rats normotendus (~10 mm Hg), mais plus significative chez les SHR (~40 mm Hg) (Mukaddam-Daher et Gutkowska, 1999, 2000 ; El-Ayoubi et coll., 2005).

L'injection aigu de la moxonidine, aux rats normotendus (SD et WKY) et hypertendus (SHR), stimule l'augmentation de la diurèse, de la natriurèse, de la kaliurèse ainsi que l'augmentation de l'excrétion de GMPc. Cependant, il y avait une différence de réponse entre les groupes suite à l'administration de la moxonidine. La diurèse stimulée par la moxonidine était moindre chez les rats SHR que chez les rats SD et WKY. La libération de l'ANP était plus élevée entre les SHR par rapport aux deux souches normotendus. Cette augmentation de la libération d'ANP serait associée à l'augmentation de la densité des récepteurs I_1 cardiaques lors de l'hypertension, car cette augmentation était observée chez les rats SHR par rapport aux deux souches normotendus (SD et WKY, où la densité des récepteurs I_1 était similaire). Les réponses rénales (natriurèse et kaliurèse) sont différentes entre SHR et WKY, mais pas entre SHR et SD. Ceci nous indique qu'en plus de la stimulation de l'ANP par les récepteurs I_1, d'autres mécanismes peuvent influencer la kaliurèse et la natriurèse. Ces mécanismes peuvent inclure la différence de la densité et de l'affinité des récepteurs I_1, α_2-adrénergiques et des récepteurs des peptides natriurétiques.

La contribution d'ANP a été confirmée par l'injection de la moxonidine après le prétraitement des rats avec l'anantin, un antagoniste des peptides natriurétiques (Weber et coll., 1991; Wyss et coll., 1991). Ces effets rénaux provoqués par la moxonidine étaient inhibés par l'anantin de façon dose-dépendante (El-Ayoubi et coll., 2005). En effet, c'est la première étude qui a signalé l'implication directe d'ANP

dans les actions rénales de la moxonidine. Conformé à notre conclusion, Smyth et coll. (2003) ont récemment rapporté que l'infusion de la moxonidine directement dans l'artère rénale gauche résulte en une excrétion d'urine et de sodium similaire de deux reins. À partir de ces résultats, ils ont suggéré l'existence d'un facteur diurétique et natriurétique extérieur qui peut être impliqué dans les effets rénaux de la moxonidine (Smyth et coll., 2003). Nous avons démontré que ce facteur diurétique et natriurétique est l'ANP.

D'autre part, lors du traitement chronique de SHR, nous avons démontré que la moxonidine diminue la pression artérielle mesurée par télémétrie. Cette diminution a été observée dès la première journée, atteignant un maximum d'effet au 4^{e} jour (Menaouar et coll., 2002) et était maintenue durant les 4 semaines de traitement (Mukaddam-Daher et Gutkowska, 2004). La moxonidine a également augmenté l'expression auriculaire et le niveau plasmatique d'ANP (Menaouar et coll., 2002). Ces études nous portent à suggérer que l'ANP soit impliqué dans les effets aigus et chroniques de la moxonidine et il sera intéressant de suggérer l'implication des récepteurs I_1 cardiaques dans la libération d'ANP.

7.3-Les peptides natriurétiques

L'ANP (28 ac.a) est un membre de la famille des peptides natriurétiques qui comprend aussi le «brain natriuretic peptide» (BNP, 32 ac.a) (Sudoh et coll., 1988), le peptide de type C (CNP, 22 et 53 ac.a) (Sudoh et coll., 1990) et l'urodilatine (De Bold et coll., 1981; Atchison et Ackermann, 1993).

L'ANP est synthétisé principalement par les oreillettes cardiaques (de Bold et coll., 1981), le BNP par les ventricules (Thibault et coll., 1992; Tateyama et coll., 1990), le CNP est produit par les cellules endothéliales (Suga et coll, 1992) et l'urodilatine est d'origine rénale.

Les formes biologiquement actives des peptides natriurétiques partagent un motif structural commun, se composant d'une boucle de 17 acides aminés constituée par une liaison bisulfure intramoléculaire entre deux résidus de cystéine (Cys^{105}-Cys^{121}) (Ruskoaho 1992; Yandle 1994; Levin et coll., 1998). Cette structure en forme d'anneau est très essentielle pour la reconnaissance par le récepteur de ces peptides et de leur fonction biologique (Inagami et coll., 1987). La queue C-terminale trouvée dans l'ANP et le BNP n'existe pas dans le CNP (Figure 4).

Les récepteurs aux peptides natriurétiques sont les récepteurs de surface qui signalent les actions physiologiques des peptides à l'intérieur des cellules cibles. À l'heure actuelle, trois classes de récepteurs ont été identifiées : le NPR-A pour "natriuretic peptide receptor type A", lie l'ANP et le BNP, le NPR-B pour "natriuretic peptide receptor type B" , lie le CNP. Le NPR-C pour "natriuretic peptide clearance receptor" lie les trois peptides et sert comme un mécanisme de clairance des peptides (Maack, 1992). Le NPR-A et le NPR-B appartiennent à la famille des guanylyl cyclases membranaires (Garbers, 1990) qui agissent par le biais de GMPc (Hamet et coll., 1984), alors que le NPR-C ne présente pas d'activité catalytique intrinsèque (Potter et Hunter, 2004 ; Koller et coll. 1991) (Figure 4). Le NPR-C inhibe également la libération de l'endothéline et antagonise le système rénine-angiotensine-aldostérone par l'intermédiaire de la

protéine Gi et l'inhibition de l'adénylyl cyclase/AMPc (Anand-Srivastava, 1992), médie la stimulation de l'oxyde nitrique endothéliale synthase (eNOS) (Murthy et coll., 2000) et active la phospholipase C (Anand-Srivastava et Trachte, 1993).

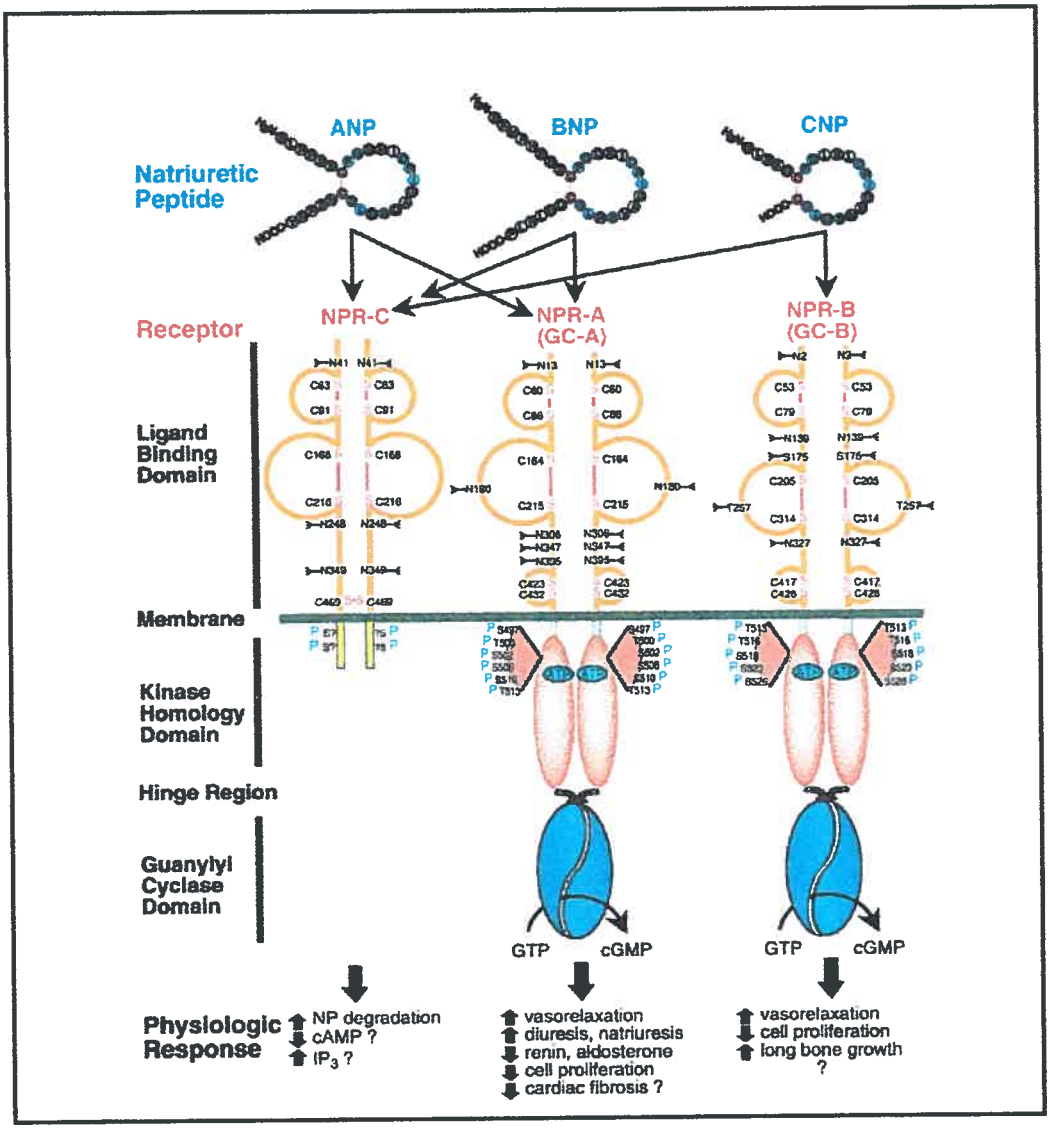


Figure 4 : Schématisation des structures et des fonctions des peptides natriurétiques et de leurs récepteurs. La numérotation correspond aux séquences de rat. Tiré de Potter et Hunter, 2001.

Les peptides natriurétiques sont des régulateurs importants de la pression artérielle et du volume sanguin en formant un lien humoral entre le cœur et les reins (Ruskoaho, 1992; Wilkins et coll., 1997; Levin et coll., 1998). L'ANP produit par le cœur agit sur les reins pour stimuler la diurèse et la natriurèse. Les effets natriurétiques et diurétiques de l'ANP sont dus à la dilatation des artérioles rénales afférentes, à la contraction des artérioles efférentes et par action directe au niveau des tubules (Loutzentiser et coll., 1988; Levin et coll., 1998). En plus, ces effets résultent de l'augmentation de la filtration glomérulaire, de la réduction de la réabsorption de sodium et de l'eau et de la suppression de rénine, d'aldostérone, de l'hormone antidiurétique et de la vasopressine (Cho et coll., 1999; Gangly, 1992). De plus, l'ANP contrecarre les effets d'angiotensine II (Espiner, 1994; Wilkins et coll., 1997; Harris et coll., 1987; Levin et coll., 1998; Jamison et coll., 1992; Johnston et coll., 1989). D'autre part, l'effet hypotenseur aigu d'ANP est principalement dû à une diminution du débit cardiaque (Atchison et Ackermann, 1993) provoquée par une réduction de volume intravasculaire (Lee et Goldman, 1989; Sugimoto et coll., 1989), par une inhibition du réflexe autonome compensatoire (Takeshita, 1990) et une inhibition de la résistance vasculaire (Ackermann, 1986), médié par l'effet sympatholytique de l'ANP (Imaizumi T et coll., 1987). Cet effet sympatholytique a été démontré chez les souris dont le gène d'ANP (-/-) ou de NPR-A (-/-) a été aboli, ils ont observé chez ces souris une augmentation de l'activité du SNS, mesurée par le niveau plasmatique des catécholamines (Melo et coll., 2000).

En plus de son effet sur la pression artérielle et le volume sanguin, les peptides natriurétiques possèdent des effets antimitotiques sur les cellules des muscles lisses. Effectivement, l'ANP a des effets antiprolifératifs dans une variété de tissus, incluant les cellules mésangiales rénales (Isono et coll., 1998), les astrocytes (Biesiada et coll.,1996), les cellules endothéliales (Morishita et coll.,1994) et les cellules vasculaires (Silberbach et Roberts, 2001).

L'ANP exerce une action antihypertrophique dans les cardiomyocytes des rats et cet effet est également exercé par les autres peptides natriurétiques (Rosenkranz et coll.,

2003). L'effet antihypertrophique de l'ANP a été mis en évidence par Oliver et coll. (1997) qui ont démontré que les souris ayant perdu le gène NPR-A (-/-) avaient une pression artérielle élevée et un phénotype marqué d'hypertrophie cardiaque avec une fibrose interstitielle cardiaque.

De plus, le système peptides natriurétiques contrecarre la prolifération et la production de la matrice extracellulaire des fibroblastes cardiaques par le biais de l'inhibition du système rénine-angiotensine et de l'endothéline (Butt et coll., 1993; Cao et Gardner, 1995), Cet effet antiprolifératif indique une implication des peptides natriurétiques dans le remodelage ventriculaire (Rubattu et Volpe, 2001). En plus de leur action en physiologie cardiovasculaire, des études ont démontré que les peptides natriurétiques, des modulateurs anti-inflammatoires de la fonction des macrophages, inhibent l'induction des médiateurs inflammatoires comme l'oxyde nitrique synthase inductible (iNOS) et le "tumor necrosis factor-alpha" (TNF-α) (Kiemer et coll., 2005; Vollmar et coll., 1994).

7.4-Implication directe des récepteurs I_1 dans la libération d'ANP

La libération d'ANP à partir des oreillettes cardiaques a comme cause primaire l'étirement de la paroi auriculaire, l'expansion du volume plasmatique (Ruskoaho, 1992). D'autres facteurs peuvent stimuler la libération d'ANP, comme l'accroissement du retour veineux par l'immersion du corps dans l'eau, l'hypoxie, l'augmentation du rythme cardiaque et de la contractilité *in vivo* et *in vitro* (Ruskoaho, 1992). En plus du rôle de l'étirement, la relâche d'ANP peut être contrôlée par des facteurs neuronaux, humoraux et vaso-actifs (l'angiotensine II, la vasopressine, l'endothéline, les prostaglandines PGF2α, PGE2 et le neuropeptide Y), les minéralocorticoïdes, les glucocorticoïdes et les hormones thyroïdiennes stimulent aussi la sécrétion d'ANP (Gardner et coll., 1987; 1988; Ruskoaho, 1992; Gardner et Schultz, 1990). La stimulation des récepteurs alpha- et béta-adrénergiques ainsi que la stimulation des récepteurs cholinergiques augmentent la sécrétion d'ANP (Ruskoaho, 1992). Par contre, l'oxyde nitrique

libéré par l'endothélium de l'endocarde pourrait réguler négativement la libération d'ANP. Des études ont montré que l'infusion des inhibiteurs de la synthèse d'oxyde nitrique (L-NAME) augmentent la sécrétion d'ANP (Sanchez-Ferrer et coll., 1990) (figure 5).

La découverte des récepteurs I_1 dans le cœur, principalement dans les oreillettes, semble très intéressante. Vu que l'activation de ces récepteurs est associée à la libération d'ANP et à ses actions et comme les oreillettes sont les sites primaires de la production d'ANP, nous avons présumé que **les récepteurs I_1 cardiaques** peuvent être impliqués dans la libération d'ANP. Puisque cette libération de l'ANP par la moxonidine peut être médiée par les mécanismes centraux et/ou périphériques, de ce fait, la participation directe des récepteurs I_1 cardiaques dans la libération d'ANP a été étudiée *in vitro* sur des cœurs isolés, en l'absence de la contribution centrale.

Des études *in vitro* ont été réalisées sur les cœurs isolés à l'aide de l'appareil de Langendorff. Dans ces études préliminaires, nous avons démontré que la perfusion des cœurs isolés des rats Sprague-Dawley et SHR avec la moxonidine augmente la libération d'ANP dosé dans l'effluent de perfusat collecté à chaque minute par rapport à celui collecté des cœurs perfusés avec le tampon. Cette libération d'ANP était plus prononcée dans le perfusat collecté des cœurs des SHR par comparaison à celui des Sprague-Dawley (Figure 1, Annexe), démontrant que les récepteurs I_1 cardiaques sont fonctionnels et qu'ils sont impliqués dans la libération d'ANP sans la contribution du système nerveux central. Par la suite, pour localiser le site cardiaque impliqué dans la libération d'ANP, des études ont été réalisées sur des sections des oreillettes et des cellules cardiaques. L'incubation des oreillettes des rats Sprague-Dawley et SHR, en présence ou non de la moxonidine à différents intervalles de temps (0 à 2 heures), révèle que la moxonidine stimule la libération d'ANP chez les oreillettes des Sprague-Dawley et SHR et cette libération était plus prononcée chez les SHR (Figure 2. Annexe). Nous avons aussi démontré que la stimulation des myocytes auriculaires des Sprague-Dawley par la moxonidine induit la libération d'ANP (Figure 3, Annexe), mais pas la stimulation des myocytes ventriculaires. Ces

résultats indiquent que 1) la moxonidine stimule la libération d'ANP du cœur, plus précisément des myocytes auriculaires; 2) cette libération était plus prononcée chez les rats SHR par rapport aux rats normotendus, cette augmentation de la libération des cœurs des SHR pouvant être expliquée par l'augmentation de la densité des récepteurs I_1 lors de l'hypertension. Aussi, la moxonidine possède une faible affinité pour les récepteurs α_2-adrénergiques, donc la libération d'ANP peut être médiée par l'activation des récepteurs I_1 et α_2-adrénergiques. D'après ces résultats, on ne peut pas distinguer l'importance de récepteurs I_1 et/ou les α_2-adrénergiques dans la libération d'ANP.

L'étude de Schafer et coll. (2002) ont démontré que la perfusion des cœurs isolés par la moxonidine réduit la libération de la norépinéphrine suite à la stimulation des récepteurs I_1 et des récepteurs α_2-adrénergiques.

En se basant sur notre étude, le groupe de Schafer ont expliqué cette inhibition par le fait que la moxonidine induit la libération d'ANP, par stimulation des récepteurs I_1 cardiaques, qui à son tour agit présynaptiquement pour inhiber l'activité sympathique et par conséquent réduit la libération de la norépinéphrine.

Cependant, les mécanismes de libération d'ANP par la moxonidine ne sont pas connus. Pourtant, Hansson et coll. (1998) ont rapporté que la sympathectomie cardiaque chimique et chirurgicale mène à une augmentation d'ANP dans les granules. La moxonidine peut affecter aussi la libération d'ANP des granules identifiés dans le système de conduction (Wharton et coll., 1988). Donc, l'augmentation d'ANP peut être expliquée en partie par l'inhibition de l'activité sympathique au cœur par la moxonidine. En plus, les seconds messagers des récepteurs I_1 (DAG, AMPc et PKC) évoquent la libération de l'ANP *in vitro*; ceci nous permet de présumer la participation des voies de signalisation des récepteurs I_1 dans la libération d'ANP. Après l'identification des récepteurs I_1 et α_2-adrénergiques dans le cœur, un autre

mécanisme impliqué dans la libération d'ANP peut s'ajouter à ceux déjà connus, par le biais de l'activation des récepteurs I_1 et α_2-adrénergiques cardiaques, plus spécifiquement au niveau des myocytes auriculaires (Figure 5).

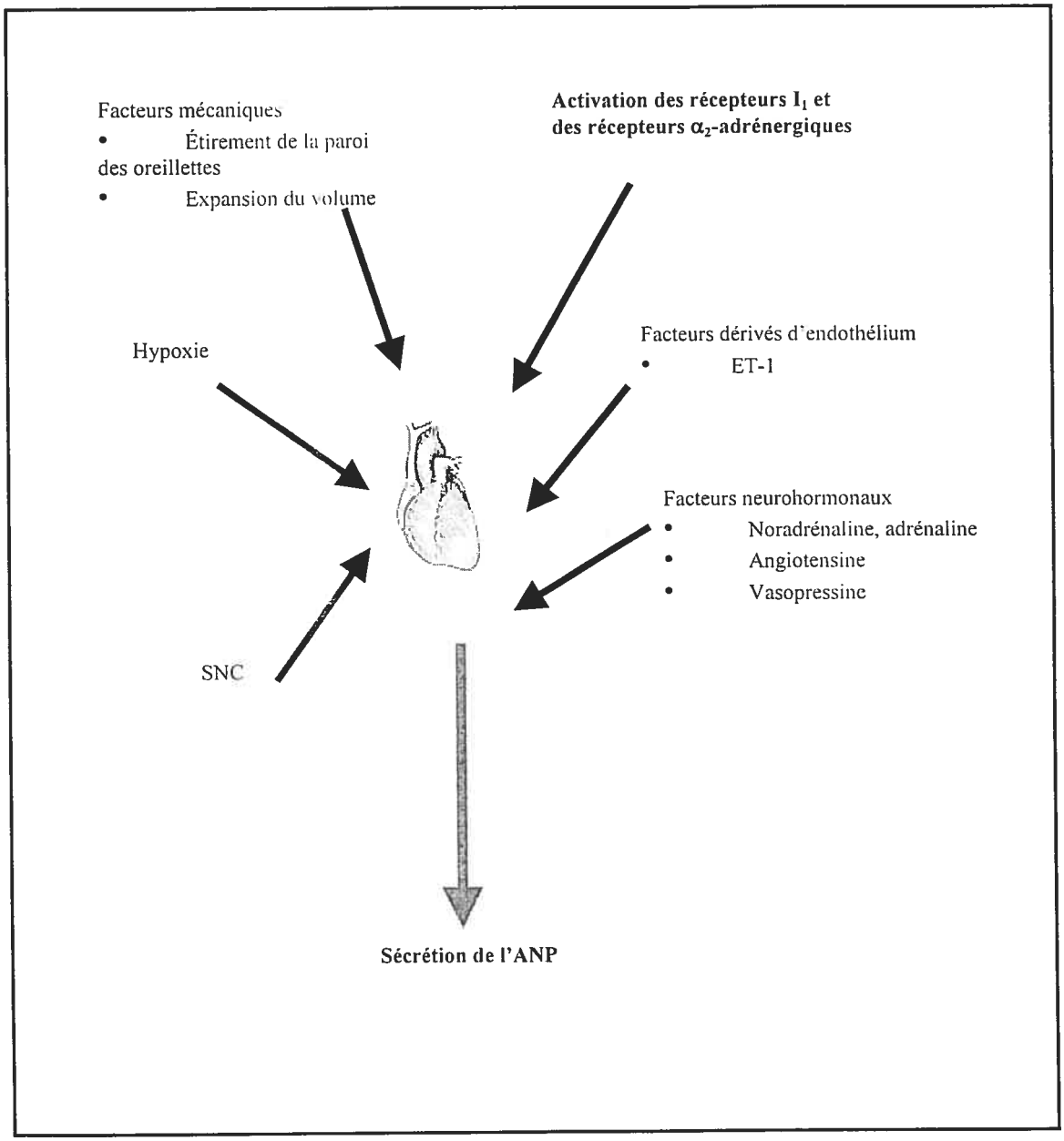


Figure 5 : les facteurs contrôlant la libération d'ANP, d'après Ruskoaho, 1992.

7.5-Signification physiologique de l'augmentation de peptides natriurétiques

Les peptides natriurétiques ont suscité un grand intérêt clinique de par leur potentiel thérapeutique. L'ANP entraîne une diminution de la pression, une réduction de la résistance vasculaire périphérique, une augmentation du volume d'éjection systolique, de la diurèse et de la natriurèse, ainsi qu'une inhibition de l'activité du SNS et du système rénine-angiotensine (Saito et coll., 1987a; Molina et coll., 1988). De nombreuses pathologies comme l'hypertension essentielle, l'insuffisance cardiaque et la défaillance rénale aiguë et chronique sont des désordres de la régulation du volume corporel caractérisées par une rétention rénale de sel et de l'eau (Brenner et coll., 1990). Ces désordres s'accompagnent de concentrations élevées d'ANP et de BNP s'expliquant par une synthèse accrue ou par une élimination diminuée des hormones, indiquant l'importance de ces hormones dans ces pathologies (Brunett et coll., 1986; Wei et coll., 1994). Une diminution de l'ANP circulant est observée lors de l'amélioration thérapeutique des désordres mentionnés ci-dessus (Marumo et coll., 1988). Ainsi, l'administration d'ANP chez les patients en insuffisance cardiaque offre des possibilités thérapeutiques très intéressantes au niveau cardiovasculaire, rénal et hormonal (Saito et coll., 1987b; Burnier et coll., 1989; Brenner et coll., 1990). L'administration d'ANP aux patients hypertendus résulte en une réduction de la pression artérielle.

En outre, les avantages du traitement des patients atteints d'insuffisance cardiaque avec l'ANP exogène et le BNP sont déjà établis. Kasama et coll. (2004) ont démontré que l'administration intraveineuse d'ANP était bénéfique pour l'activité du nerf sympathique cardiaque et qu'elle a amélioré le remodelage du ventricule gauche chez ces patients. L'administration du BNP exogène a été développée comme nouveau traitement pour l'insuffisance cardiaque congestive et était approuvée aux États-Unis. Les nouvelles stratégies thérapeutiques pour l'insuffisance cardiaque incluent actuellement l'administration sous-cutanée chronique et l'infusion intermittente de BNP.

Une stratégie alternative sera l'utilisation des drogues qui augmentent le niveau endogène des peptides natriurétiques. Des études ont démontré que l''effet de l'iECA a été renforcé lors de l'inhibition de l'endopeptidase neutre (Seymour et coll., 1995), un inhibiteur du métabolisme des peptides natriurétiques (Marleau et coll., 1990; Erdos et Skidgel, 1989) et de ce fait augmente leurs niveaux endogènes. L'omapatrilat, cumulant également une activité d'inhibiteur de l'ECA et de l'endopeptidase, est utilisée pour le traitement de l'insuffisance cardiaque et l'hypertension. Malheureusement, cette utilisation de l'omapatrilat était limitée chez les patients atteints d'insuffisance cardiaque du fait qu'elle provoque l'augmentation de l'endothéline plasmatique (Elmarakby et coll., 2003).

Ainsi, nos études ont démontré que le traitement aigu à la moxonidine augmente le niveau plasmatique d'ANP et le traitement chronique (une semaine) augmente l'expression cardiaque et le niveau plasmatique d'ANP. Vu que l'activation des récepteurs aux imidazolines par la moxonidine était associée à une augmentation de la synthèse et de la libération d'ANP ceci pourrait signaler une autre approche alternative pour stimuler les peptides natriurétiques endogènes.

Cette augmentation d'ANP par la moxonidine peut jouer un rôle important dans la régulation chronique de la pression artérielle. L'évidence définitive de l'implication d'ANP dans la régulation chronique de la pression artérielle est dérivée des études génétiques sur divers modèles de souris transgéniques. Les souris transgéniques surexprimant le gène ANP (TTR-ANP) sont hypotendus de 25-30 mm Hg relativement aux animaux témoins (Steinhelper, 1990), tandis que les souris dont le gène d'ANP ou de récepteur NPR-A a été aboli (knock-out) (John et coll., 1995; Oliver et coll., 1997) sont hypertendus de 20-30 mm Hg par rapport aux contrôles. Ces souris transgéniques ont permis de fournir des évidences complémentaires de l'effet hypotendu d'ANP. En plus, nous avons montré aussi l'augmentation de l'expression auriculaire et du niveau plasmatique de BNP pendant une semaine et la normalisation durant quatre semaines du traitement chronique à la moxonidine.

Cette augmentation de BNP semble être également très importante dans notre étude, en raison de l'implication de BNP dans la réduction de la fibrose (Tamura et coll., 2000). Chez les souris knock-out pour le gène de BNP, aucun signe d'hypertension ou d'hypertrophie ventriculaire n'était observé, mais plusieurs régions cardiaques ont été atteintes de fibrose, résultant des interactions complexes entre les myocytes et les non-myocytes. Compte tenu que l'hypertension artérielle représente un facteur de risque pour les complications fibrotiques, l'augmentation de BNP dans notre étude paraît très importante, vu que le BNP est un facteur antifibrotique et un régulateur local du remodelage ventriculaire.

Diverses études ont démontré que le traitement à la moxonidine réduit l'hypertrophie ventriculaire gauche chez les patients et les rats (Mall et coll., 1991; Ollivier et Christen, 1994). Chez les rats après infarctus du myocarde, le traitement à la moxonidine inhibe l'activation sympathique, prévient l'hypertrophie cardiaque et reconstitue le contenu interstitiel de collagène jusqu'aux valeurs normales (van Zwieten, 1997). Chez les SHR, la moxonidine normalise la fibrose myocardique et la capillarisation aux niveaux physiologiques (Mall et coll., 1991). Dans notre étude, les SHR (12-14 semaines) possèdent un ratio de masse ventriculaire gauche par rapport au poids corporel (VG/PC) plus élevé que les rats normotendus, indiquant la présence de l'hypertrophie ventriculaire gauche chez les SHR. Par conséquent, la régression de l'hypertrophie ventriculaire est considérée comme un effet souhaitable du traitement antihypertenseur. Nous avons démontré que le ratio VG/PG tend à diminuer après le traitement chronique à la moxonidine d'une semaine (Menaouar et coll., 2002) et a été normalisé significativement après un mois de traitement (Mukaddam-Daher et Gutkowska, 2004). Cette régression de l'hypertrophie ventriculaire gauche peut être influencée en partie par l'ANP.

L'hypertrophie prolongée peut contribuer au dysfonctionnement cardiaque menant à l'insuffisance cardiaque et à la mort soudaine. En conséquence, l'utilisation des drogues potentielles pour les peptides natriurétiques endogènes, telles des

agonistes sélectifs pour les récepteurs I_1 et des inhibiteurs pharmacologiques du métabolisme des peptides natriurétiques, sont des alternatives thérapeutiques attirantes. En outre, l'augmentation de l'expression et du taux plasmatique de l'ANP et de BNP, après une semaine de traitement, pourrait être expliquée par des effets autocrines/paracrines de compensation pour défendre contre la progression de l'hypertrophie maladaptive, de fibrose et pour protéger le cœur. Il s'avère probablement que la diminution de la pression artérielle et la régression de l'hypertrophie ventriculaire sont expliquées en partie par l'activation des récepteurs I_1 cardiaques et leur implication dans la libération des peptides natriurétiques. En outre, la moxonidine présente des avantages plus importants que la régulation de la pression artérielle par leurs effets cardioprotecteurs, antiprolifératifs, anti-arythmiques (Regunathan et coll., 1999; Mall et coll., 1991) qui pourraient être expliqués en partie par l'ANP.

D'autres études devraient être réalisées pour étudier le mécanisme impliqué dans l'effet cardioprotecteur des récepteurs aux imidazolines et la participation de l'ANP et de BNP aussi bien au niveau biochimique que moléculaire.

7.6-Importance des récepteurs I_1 cardiaques, nouvelles cibles pour les agents antihypertenseurs

La multitude d'études cliniques randomisées menées avec les imidazolines attestent de leur efficacité du point de vue de la protection contre les événements cardiovasculaires chez les patients hypertendus.

L'utilisation chronique des agents imidazoliniques sélectifs pour les récepteurs aux imidazolines, est associée à des effets bénéfiques additionnels à leurs effets hypotenseurs. Ces effets bénéfiques sont caractérisés entre autres par une régression efficace de l'hypertrophie cardiaque, des infarctus et de la dysfonction systolique/diastolique, etc.

Comme ces effets bénéfiques ne peuvent s'expliquer uniquement par un contrôle plus efficace de la tension artérielle, il est permis de postuler que ces effets bénéfiques cliniques sont reliés à l'inhibition de l'activité sympathique et au blocage de certains effets médiés par l'Ang II et l'épinéphrine.

Dans ce contexte, nos travaux ont permis de démontrer pour la première fois l'implication des récepteurs I_1 cardiaques dans les effets bénéfiques des imidazolines suite à leur capacité de produire l'ANP. Le fait que les composés imidazolines possèdent le pouvoir de libérer l'ANP, ceci amplifie les effets bénéfiques de ces composés et nous incite à élucider dans le futur proche les mécanismes impliqués dans la libération d'ANP, dans le but de mettre au point un traitement efficace contre l'hypertension et ses complications. Néanmoins, il est permis de spéculer que les effets bénéfiques de la moxonidine pourraient être expliqués, du moins en partie, par un effet potentiel relayé par les récepteurs I_1 cardiaques et que ces récepteurs pourraient représenter une nouvelle cible thérapeutique pour le contrôle du remodelage cardiovasculaire lors de l'hypertension.

Conclusion

Les approches pharmacologiques utilisées dans ces études ont clairement démontré pour la première fois la présence des récepteurs I_1 et α_2-adrénergiques dans le cœur et la régulation des récepteurs I_1 cardiaques lors des désordres cardiavasculaires. L'activation aiguë et chronique des récepteurs I_1 et α_2-adrénergiques par la moxonidine est associée à l'augmentation de l'ANP. En plus, nous avons démontré que les récepteurs cardiaques sont impliqués directement dans la libération de l'ANP et que cet effet est indépendant des effets hémodynamiques et centraux.

Finalement, la moxonidine possède le pouvoir de réduire la pression sanguine, de stimuler l'excrétion de sodium et de l'eau, de stimuler la libération d'ANP et de normaliser l'hypertrophie ventriculaire qui représente une condition pathologique de l'hypertension. Tous les effets de la moxonidine mentionnés ci-dessus peuvent être médiés par l'activation des récepteurs I_1 centraux, rénaux et on peut ajouter un autre mécanisme qui est l'activation des récepteurs I_1 cardiaques (Figure 6). En conclusion, ces résultats apportent des éléments en faveur de l'implication des récepteurs I_1 cardiaques avec l'ANP dans la médiation des effets hémodynamiques et rénaux de la moxonidine. Il existe encore des nombreuses lacunes dans les connaissances actuelles sur les mécanismes moléculaires déterminant la diminution de la pression artérielle et la régression de l'hypertrophie cardiovasculaires en réponse à des agents antihypertenseurs, qui est un sujet très important tant du point de vue fondamental que clinique. Le développement de nouvelles thérapies dépendra d'une meilleure compréhension des mécanismes impliqués dans la libération d'ANP, de même que le rôle pathophysiologique des récepteurs I_1 et α_2-adrénergiques cardiaques.

Toutefois, bien que nos travaux fournissent des informations fondamentales sur la présence des récepteurs I_1 cardiaques et de leur implication dans la libération d'ANP,

plusieurs questions doivent être prises en considération. En effet, que se passe-t-il chez les souris knock-out pour les récepteurs I_1 traités à la moxonidine ? Que donne le traitement à la moxonidine chez les souris knock-out pour les récepteurs ou le gène d'ANP ?

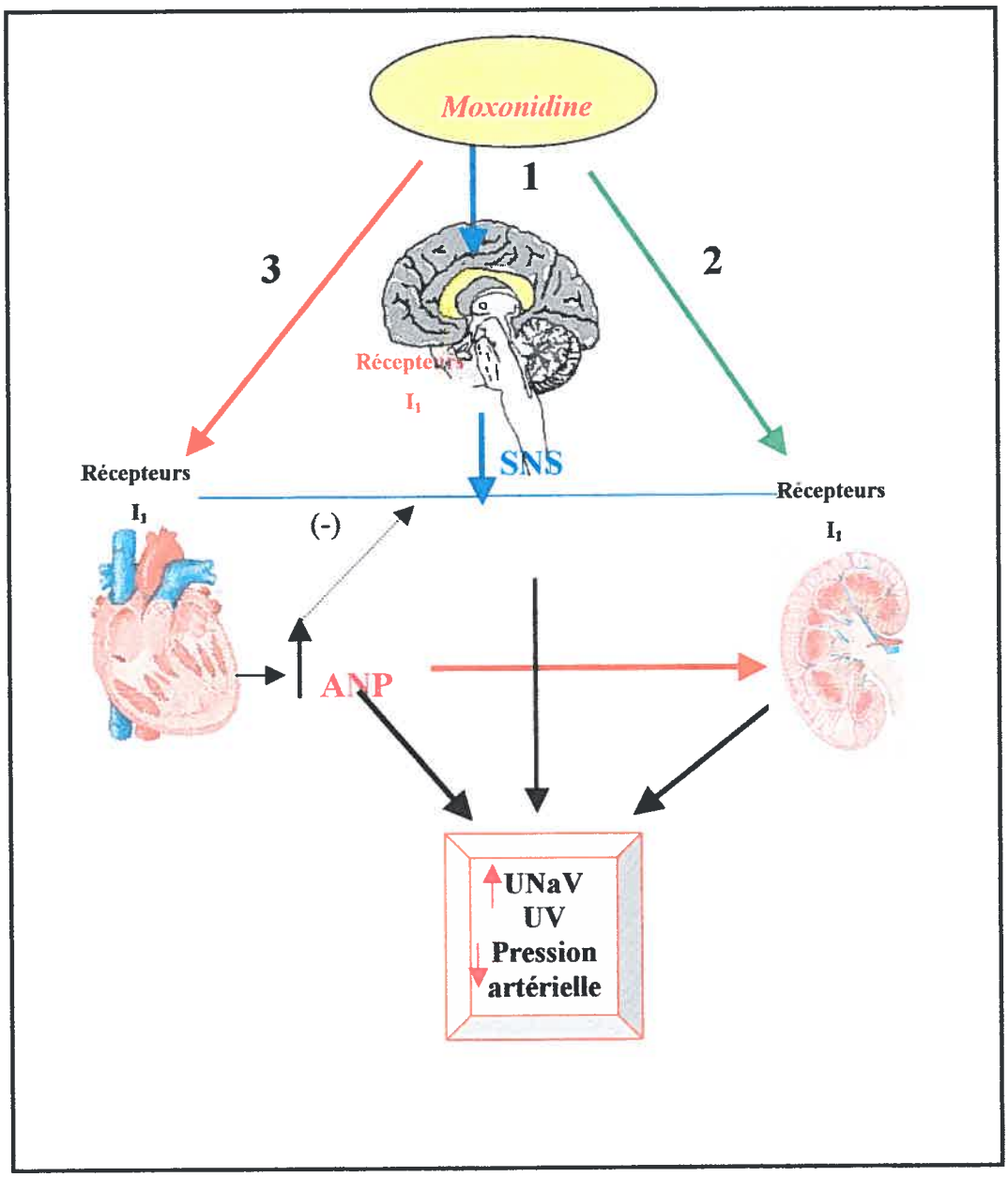


Figure 6 : les mécanismes de réduction de la pression artérielle par la moxonidine par l'activation des récepteurs aux imidazolines I_1.

1-Activation des récepteurs I_1 centraux et inhibition de l'activité de SNS ; 2-Activation des récepteurs I_1 rénaux et stimulation de la diurèse, de la natriurèse et de la kaliurèse ; 3-Activation des récepteurs I_1 cardiaques et stimulation de la libération d'ANP qui à son tour: inhibe l'activité du SNS au rein, stimule la diurèse, la natriurèse, la kaliurèse et l'excrétion de GMPc par actions sur ces récepteurs.

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ANNEXE

Effet de l'activation des récepteurs I_1 par la moxonidine sur la libération d'ANP

I-Cœur isolé de rat

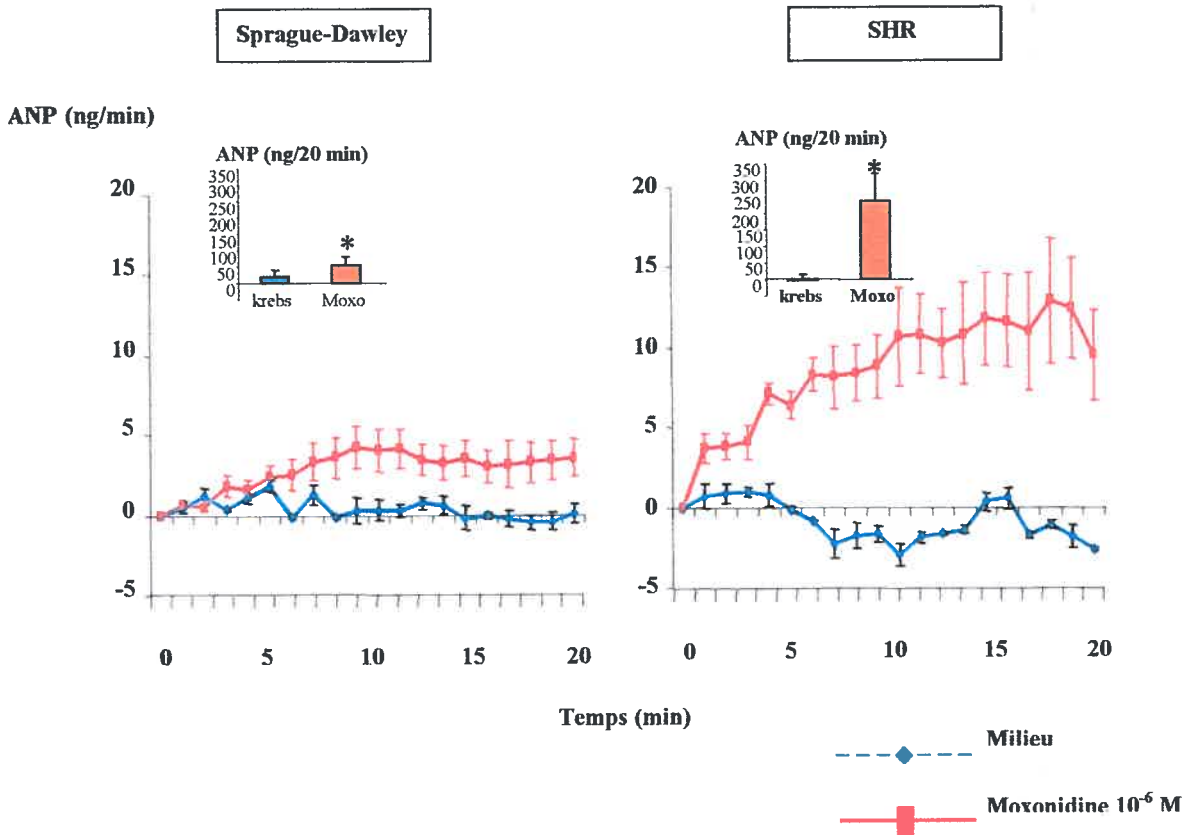

Figure 1: Des études *in vitro* ont été réalisées sur les cœurs isolés à l'aide de l'appareil de langendorff afin de montrer l'implication directe des récepteurs I_1 dans la libération d'ANP. La moxonidine (10^{-6}M) a stimulé la libération de peptide natriurétique auriculaire (ANP), dosé par radioimmunoassay (RIA) dans l'effluent (collecté chaque minute), des coeurs isolés des rats Sprague-Dawley et SHR perfusés avec le tampon de krebs en présence ou non de la moxonidine. Cette libération d'ANP était plus prononcée dans le perfusat collecté des cœurs des SHR par comparaison à celui des Sprague-Dawley perfusés à la moxonidine. *P<0,002 par rapport à la quantité libérée par les cœurs perfusés avec le tampon seul.

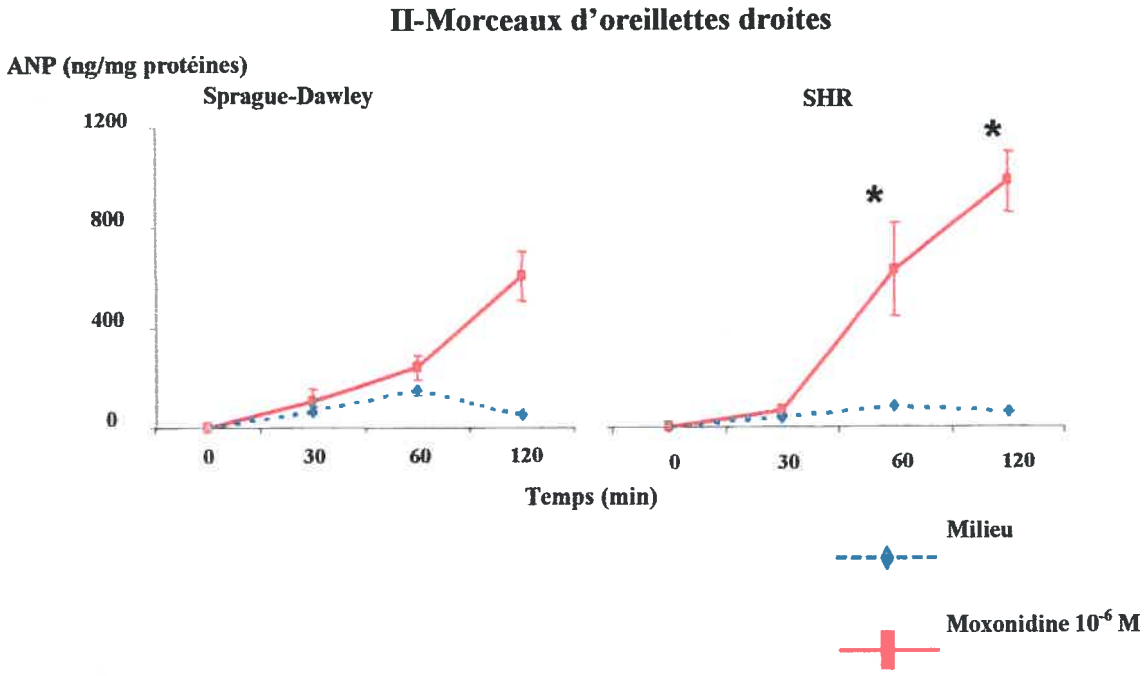

Figure 2: Des sections des oreillettes droites des rats Sprague-Dawley et SHR étaient incubées en présence ou non de la moxonidine (10^{-6} M) pendant 2 heures. Des échantillons (50µl) étaient collectés à différentes échelles du temps (0, 30, 60 et 120min) pour le dosage d'ANP par RIA. La moxonidine a induit la libération d'ANP, des sections des oreillettes droites, des rats Sprague-Dawley et SHR. La moxonidine augmente la libération d'ANP de façon significative après 2h d'incubation chez les Sprague-Dawley et à 60 minutes chez les SHR. Cette libération était plus prononcée dans les échantillons collectés des oreillettes des rats SHR par rapport aux rats Sprague-Dawley. *P<0,02 par rapport aux oreillettes stimulés par le milieu.

III-Cardiomyocytes auriculaires en Culture

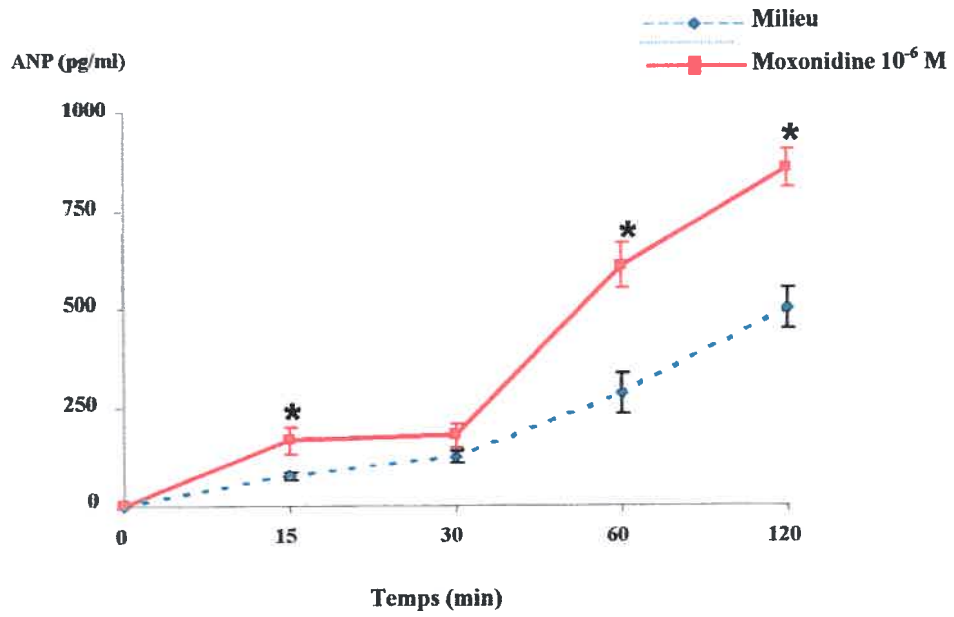

Figure 3: La moxonidine a stimulé la libération d'ANP, dosé par RIA dans des échantillons collectés à différents intervalles de temps (0 à 2 h) suite à l'incubation des myocytes néonataux auriculaires, des rats Sprague-Dawley, en présence ou non de la moxonidine (10^{-6} M). Cette libération d'ANP était significativement augmentée à 15, 60 et 120 minutes par rapport aux myocytes stimulés par le milieu du culture. La libération d'ANP n'était pas observée après la stimulation des myocytes néonataux ventriculaires des Sprague-Dawley par la moxonidine. *P<0.02 par rapport à la quantité libérée par les myocytes stimulés par le milieu.

Curriculum Vitae

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FORMATION

Jan 2001-nov 2005	Ph.D. en sciences Biomédicales, Université de Montréal
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Jan1996-dec1999	Baccalauréat en Biologie Moléculaire Université de Québec à Montréal
Sep1994-juin 1995	Apprentissage de l'anglais, Université de Toronto, Ontario.

COMPÉTENCES TECHNIQUES

- Purification et analyses des protéines (HPLC, électrophorèse, Western blot, RT-PCR)

- Radio-marquage, dosage radio-immunologique et dosage ELISA.

- Culture cellulaire

- Cours de formation en radio-protection

- Churigie aseptique des animaux.

- Cours théorique et pratique sur la manipulation des animaux de laboratoire.

- Techniques pour détecter l'apoptose

- Maîtrise des logiciels Word, Excel, Power point et Internet.

BOURSES D'EXCELLENCE

- Bourse d'excellence du Gouvernement canadien en Janvier 2000 (Fondation Canadienne des Bourses d'études du millénaire).
- Prix d'excellence pour meilleure présentation (poster) au 4eme journée scientifique des étudiants et stagiaires du CHUM; Montréal, Québec (déc. 2001).
- Prix d'excellence pour meilleure présentation (poster) Journées de la Recherche; Congrès annuel des étudiants de l'AEGSFM: Université de Montréal (Jan. 2002).
- Bourse d'excellence pour le dossier académique en Sciences Biomédicales et en recherche. Université de Montréal (décembre 2003).
- Bourse de rédaction en Sciences Biomédicales et en recherche. Université de Montréal (septembre 2004).
- Bourse des études post-doctorales du conseil de recherches en sciences naturelles et en génie du Canada (en septembre 2005)

CONTRIBUTIONS À LA RECHERCHE

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38. **El-Ayoubi R**., Menaouar A, Gutkowska J, Mukaddam-Daher S: Atrial natriuretic peptide (ANP) is directly involved in the renal actions of moxonidine. 24th Annual Meeting of the Canadian Hypertension Society, Toronto (Oct. 2003).

39. **El-Ayoubi R**, Duguay D, Menaouar A, DeBlois D, Gutkowska J, Mukaddam-Daher S: Regression of left ventricular hypertrophy by moxonidine. 24th Annual Meeting of the Canadian Hypertension Society, Toronto (Oct. 2003).

40. Menaouar A, **El-Ayoubi R**, Gutkowska J, Mukaddam-Daher S: Cardiac Effects of Chronic Moxonidine Treatment in Spontaneously Hypertensive Rats. 24th Annual Meeting of the Canadian Hypertension Society, Toronto (Oct. 2003).

41. Menaouar A, **El-Ayoubi R**, Gutkowska J, Mukaddam-Daher S. Effets cardiovasculaires du traitement chronique des rats spontanément hypertendus avec la moxonidine. 6eme journée scientifique des étudiants et stagiaires du CHUM; Montréal, Québec (Dec. 2003).

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Urinary responses to acute moxonidine are inhibited by natriuretic peptide receptor antagonist

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1 We have previously shown that acute intravenous injections of moxonidine and clonidine increase plasma atrial natriuretic peptide (ANP), a vasodilator, diuretic and natriuretic hormone. We hypothesized that moxonidine stimulates the release of ANP, which would act on its renal receptors to cause diuresis and natriuresis, and these effects may be altered in hypertension.

2 Moxonidine (0, 10, 50, 100 or 150 μg in 300 μl saline) and clonidine (0, 1, 5 or 10 μg in 300 μl saline) injected intravenously in conscious normally hydrated normotensive Sprague–Dawley rats (SD, ~200 g) and 12–14-week-old Wistar-Kyoto (WKY) and spontaneously hypertensive rats (SHR) dose-dependently stimulated diuresis, natriuresis, kaliuresis and cGMP excretion, with these effects being more pronounced during the first hour post-injection. The actions of 5 μg clonidine and 50 μg moxonidine were inhibited by yohimbine, an α_2-adrenoceptor antagonist, and efaroxan, an imidazoline I_1-receptor antagonist.

3 Moxonidine (100 μg) stimulated ($P<0.01$) diuresis in SHR (0.21 ± 0.04 vs 1.16 ± 0.06 ml h^{-1} 100 g^{-1}), SD (0.42 ± 0.06 vs 1.56 ± 0.19 ml h^{-1} 100 g^{-1}) and WKY (0.12 ± 0.04 vs 1.44 ± 0.21 ml h^{-1} 100 g^{-1}). Moxonidine-stimulated urine output was lower in SHR than in SD and WKY. Moxonidine-stimulated sodium and potassium excretions were lower in SHR than in SD, but not WKY, demonstrating an influence of strain but not of pressure. Pretreatment with the natriuretic peptide antagonist anantin (5 or 10 μg) resulted in dose-dependent inhibition of moxonidine-stimulated urinary actions. Anantin (10 μg) inhibited ($P<0.01$) urine output to 0.38 ± 0.06, 0.12 ± 0.01, and 0.16 ± 0.04 ml h^{-1} 100 g^{-1} in SD, WKY, and SHR, respectively. Moxonidine increased ($P<0.01$) plasma ANP in SD (417 ± 58 vs 1021 ± 112 pg ml^{-1}) and WKY (309 ± 59 vs 1433 ± 187 pg ml^{-1}), and in SHR (853 ± 96 vs 1879 ± 229 pg ml^{-1}).

4 These results demonstrate that natriuretic peptides mediate the urinary actions of moxonidine through natriuretic peptide receptors.

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Keywords: Moxonidine; clonidine; natriuretic peptides; imidazoline receptors; α_2-adrenoceptors; natriuresis; cGMP; SHR; anantin

Abbreviations: ANP, atrial natriuretic peptide; BNP, brain natriuretic peptide; I_1-receptors, imidazoline I_1-receptors; NPR-A, natriuretic peptide receptor-A; SD, Sprague–Dawley; SHR, spontaneously hypertensive rats; WKY, Wistar–Kyoto

Introduction

Centrally acting antihypertensive compounds, such as clonidine, moxonidine, and rilmenidine, reduce blood pressure by acting, albeit with different affinities, on α_2-adrenoceptors and imidazoline I_1-receptors (I_1-receptors), resulting in sympathoinhibition. In addition, these drugs may directly act on the kidneys to stimulate diuresis and natriuresis, thus contributing to short- and long-term control of blood pressure (Ziegler *et al.*, 1996; Ernsberger, 2000).

Several groups have investigated the mechanisms involved in the renal responses to acute injections of these centrally acting compounds. Smyth & Penner (1998) have shown that intracerebroventricular administration of moxonidine produces significant increases in urine and sodium excretion that are totally blocked by intravenous prazosin, implicating inhibition of renal nerve activity and subsequent α_1-adrenoceptor stimulation in these effects. Further studies have shown that, independent of the renal nerves and vasopressin, moxonidine may exert direct effects on the kidney to cause diuresis and natriuresis (Allan *et al.*, 1993; Smyth & Penner, 1998), by acting on its receptors in the proximal tubules (Bidet *et al.*, 1990; Limon *et al.*, 1992; Li & Smyth, 1993a; Bohmann *et al.*, 1994; Greven & von Bronewski-Schwarzer, 2001) to inhibit the Na^+–H^+ exchanger (Schlatter *et al.*, 1997).

Comparing two imidazoline compounds with different affinities for I_1-receptors vs α_2-adrenoceptors, Hohage *et al.* (1997b) reported that intravenous moxonidine, which binds to I_1-receptors with greater affinity than clonidine, transiently increased fractional fluid and sodium excretion in anesthetized rats, whereas equal concentrations of clonidine resulted in a sustained increase in fractional fluid excretion. The effects were inhibited by selective antagonists, leading to the

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conclusion that the drugs acted on two different receptors (Hohage *et al*., 1997b).

In contrast, Hohage *et al.* (1997a) demonstrated, in spontaneously hypertensive rats (SHR), that moxonidine, but not equal concentrations of clonidine, stimulated diuresis and natriuresis, and that the effects were long-lasting, suggesting altered response in hypertension. However, the influence of hypertension was not investigated, as the study did not include normotensive controls. Li *et al.* (1994) reported that the renal effects of intravenous moxonidine were attenuated in 1 kidney-1 clip (1K1C) hypertensive rats compared to sham controls, and attributed the attenuation to downregulation of renal imidazoline receptors in this model (Li & Smyth, 1993a).

In earlier studies, we have demonstrated that acute intravenous injections of various doses of clonidine and moxonidine in normotensive Sprague–Dawley (SD) rats evoked dose-dependent diuresis and natriuresis, and contrary to the finding of Hohage *et al.* (1997a, b), clonidine was at least 10 times more potent that moxonidine. The renal effects were inhibited by yohimbine as well as by efaroxan, a selective imidazoline I_1-receptor antagonist (Mukaddam-Daher & Gutkowska, 2000). We also reported preliminary results (Mukaddam-Daher & Gutkowska, 1999) from SHR and Wistar–Kyoto (WKY) rats, showing that moxonidine significantly stimulated diuresis and natriuresis and that the effects were totally inhibited by efaroxan and partially by yohimbine, indicating that the effects of moxonidine were primarily mediated by imidazoline receptors. Other experiments revealed that the efaroxan dose used in that study (500 μg per rat) was too high to correctly draw a conclusion on the receptor type mediating these effects. Our investigations, however, revealed that acute treatment with clonidine and moxonidine was associated with a dose-dependent increase of plasma atrial natriuretic peptide (ANP) and urinary excretion of cGMP, an index of natriuretic peptide activity. ANP is a potent vasodilator, diuretic and natriuretic hormone, primarily of cardiac origin. Accordingly, we proposed a new mechanism of action of moxonidine, namely, the involvement of natriuretic peptides in these effects. Direct proof of this hypothesis remained to be performed.

Owing to controversial reports on the mechanisms and receptor type(s) involved in the renal effects of imidazoline compounds, and regulation in hypertension, the present study was designed to examine renal responses to acute intravenous moxonidine and clonidine in conscious hypertensive rats (SHR) compared to two normotensive models, SD and WKY rats. The direct involvement of natriuretic peptides in these effects was demonstrated by using anantin, the first microbially produced competitive peptide antagonist of natriuretic peptides. At doses that do not evoke agonistic effects, anantin dose-dependently inhibits ANP-induced intracellular cGMP accumulation in bovine aorta smooth muscle cells (Weber *et al.*, 1991; Wyss *et al.*, 1991).

Methods

Female SHR (12–14 weeks old) and age-matched normotensive WKY, as well as normotensive SD rats (~200 g) purchased from Charles River (St-Constant, Quebec, Canada), were housed in a temperature- and light-controlled room with free access to food and water. The two normotensive control groups were used to investigate the selective effect of blood pressure without the confounding influence of genetic background. Experiments were approved by the Animal Care Committee of the CHUM, according to the Canadian Council on Animal Care guidelines.

All experiments were started in the morning (around 08:00 h). One dose of moxonidine (0, 1, 10, 50, 100 or 150 μg) or the reference drug clonidine (0, 1, 5, or 10 μg) was injected into the tail vein in different groups. The injection procedure took about 60 s. Then, the rats were placed individually in Nalgene plastic metabolic cages (Braintree Scientific, Inc., Braintree, MA, U.S.A.) without food and water. Spontaneously voided urine was collected every hour, over four consecutive hours, for the measurement of urine volume and electrolyte excretion. In other experiments, rats were injected with 50 μg moxonidine or 5 μg clonidine after 10-min pretreatment with efaroxan (250 or 25 μg) or yohimbine (50 or 25 μg) in 300 μl saline.

The contribution of natriuretic peptides to the urinary effects of 100 μg moxonidine was investigated in separate groups of rats, after 10-min pretreatment with anantin (5 or 10 μg per rat). The anantin doses were chosen in preliminary experiments on normotensive SD rats and shown to dose-dependently inhibit diuresis and natriuresis, as well as cGMP excretion, evoked by acute volume expansion (by rapid injection of 6 ml isotonic saline), the primary stimulus of natriuretic peptide release.

Separate groups of rats were killed by decapitation 15–20 min after injection of moxonidine (100 μg) or an equal volume of saline vehicle. Blood was collected in prechilled tubes containing protease inhibitors in a final concentration: 1 mmol l^{-1} EDTA, 5 μmol l^{-1} Pepstatin A, and 10 μmol l^{-1} phenylmethylsulfonyl fluoride (PMSF), and immunoreactive ANP was measured in extracted plasma by a specific radioimmunoassay as described previously (Gutkowska, 1987). Urinary cGMP was quantified by radioimmunoassay according to a previously described method (Gutkowska *et al.*, 1997). Urinary sodium and potassium concentrations were measured with a flame photometer (Perkin-Elmer 51, Norwalk, CT, U.S.A.), and excretions per hour were calculated. Although the rats had almost similar body weight (~200 g), their renal parameters were normalized to percent body weight to avoid the effect of any body weight variation among the different groups.

Drugs

Moxonidine (kindly provided by Solvay Pharmaceuticals, Hannover, Germany) was dissolved in 0.1 mol l^{-1} acetic acid, and its volume was brought up to the required concentration with normal saline. Clonidine, efaroxan, and yohimbine (Sigma-Aldrich, St Louis, MO, U.S.A.) were dissolved in saline. Anantin (Cedarlane Laboratories Ltd, Hornby, ON, Canada) was dissolved in 50% acetic acid, aliquoted, and stored at −20°C. On the day of the experiment, aliquots were diluted to 5 or 10 μg in 300 μl saline. All solutions were freshly prepared on the day of the experiments.

Data analysis

Statistical analysis of data obtained from normotensive SD and WKY rats and hypertensive SHR with and without

different treatments was performed by ANOVA, followed by Neuman–Keuls multiple comparison test. $P<0.05$ was considered significant. All data are expressed as mean±s.e.m.

Results

Renal parameters measured over 4 h post-moxonidine and clonidine in normotensive SD and WKY rats and hypertensive SHR revealed that the effects were more pronounced during the first hour of treatment. First hour urine output after injection of saline vehicle was higher in SD ($0.42\pm0.06\,ml^{-1}\,h^{-1}$ $100\,g^{-1}$ body weight, $n=13$, $P<0.001$) than in WKY ($0.12\pm0.04\,ml^{-1}\,h^{-1}$ $100\,g^{-1}$, $n=26$) and SHR ($0.21\pm0.04\,ml^{-1}\,h^{-1}$ $100\,g^{-1}$, $n=16$). Increasing doses of clonidine (Figure 1) and moxonidine (Figure 2) stimulated the excretion of urine, sodium, potassium, and cGMP in a dose-dependent manner, with similar profiles.

The renal responses to 5 μg clonidine and 50 μg moxonidine and inhibition by yohimbine and efaroxan are depicted in Figures 3 and 4. Yohimbine at 25 μg (data not shown) tended to, but did not significantly suppress, the renal parameters evoked by clonidine and moxonidine. At 50 μg, the inhibitory effect of yohimbine was more evident. Efaroxan at 25 μg *significantly* inhibited and at 250 μg (data not shown) it *totally* suppressed both clonidine- and moxonidine-stimulated renal parameters. Thus, α_2-adrenoceptors and I_1-receptors are implicated in these renal responses.

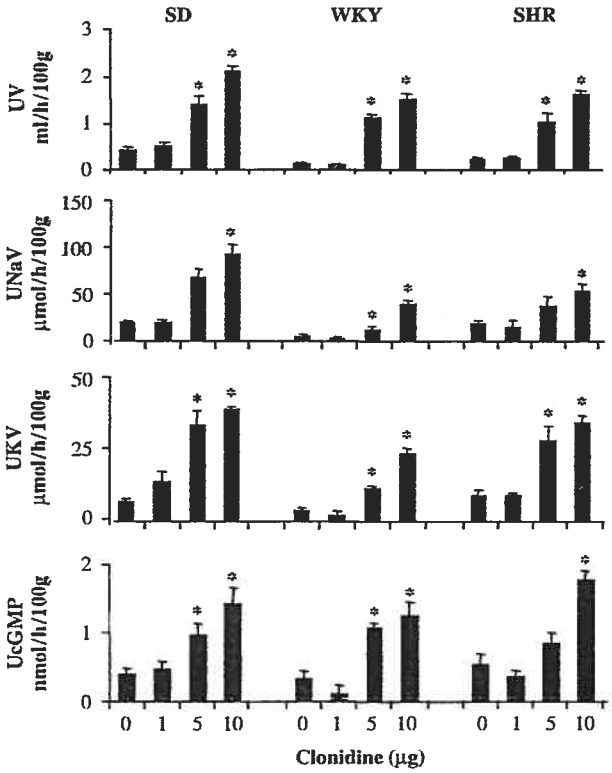

Figure 1 Effect of increasing doses of clonidine on urine output (UV, ml h^{-1} 100 g^{-1}), sodium (UNaV, μmol h^{-1} 100 g^{-1}), potassium (UKV, μmol h^{-1} 100 g^{-1}), and cGMP (UcGMP, nmol h^{-1} 100 g^{-1}) excretions during the first hour of drug administration in SD, WKY and SHR ($n=5$–32 rats per group per treatment). *$P<0.001$ vs corresponding saline control.

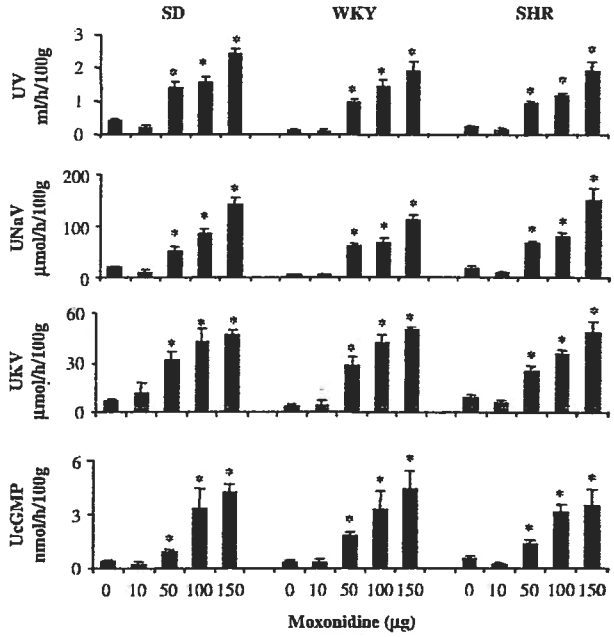

Figure 2 Effect of increasing doses of moxonidine on urine output, sodium, potassium, and cGMP excretions during the first hour of drug administration in SD, WKY, and SHR ($n=5$–30 rats per group per treatment). *$P<0.001$ vs corresponding saline control.

Compared to saline vehicle, moxonidine at 100 μg significantly ($P<0.001$) increased the renal parameters measured over 1 h post-injection in all groups. Moxonidine-stimulated urine output in SHR ($1.16\pm0.06\,ml^{-1}\,h^{-1}$ $100\,g^{-1}$, $n=16$) was significantly ($P<0.05$) lower than in SD ($1.56\pm0.19\,ml^{-1}\,h^{-1}$ $100\,g^{-1}$, $n=10$) and WKY rats ($1.44\pm0.21\,ml^{-1}\,h^{-1}$ $100\,g^{-1}$, $n=12$) (Figure 5). Sodium and potassium excretions were also significantly ($P<0.05$) lower in SHR compared to SD but not WKY rats.

Figure 5 also shows that pretreatment with the natriuretic peptide receptor (NPR) antagonist, anantin, dose-dependently inhibited the first hour renal parameters stimulated by moxonidine. At 10 μg, anantin inhibited ($P<0.001$) moxonidine-stimulated urine output to $0.38\pm0.06\,ml^{-1}\,h^{-1}$ $100\,g^{-1}$ in SD rats and to 0.12 ± 0.01 and $0.16\pm0.04\,ml^{-1}\,h^{-1}$ $100\,g^{-1}$ in WKY and SHR, respectively. Similarly, anantin totally abolished moxonidine-stimulated sodium, potassium, and cGMP excretions (Figure 5).

Plasma ANP levels measured 15–20 min after moxonidine or saline-vehicle injections are shown in Figure 6. Plasma ANP tended to be higher in SHR ($853\pm96\,pg\,ml^{-1}$, $n=14$) than in WKY ($309\pm59\,pg\,ml^{-1}$, $n=5$) and SD ($417\pm58\,pg\,ml^{-1}$, $n=18$) rats. Moxonidine stimulated plasma ANP in SHR ($1878\pm229\,pg\,ml^{-1}$, $n=14$, $P<0.001$) and WKY ($1433\pm187\,pg\,ml^{-1}$, $n=5$, $P<0.01$) to higher levels than corresponding SD ($1021\pm112\,pg\,ml^{-1}$, $n=19$).

Discussion

The results of the present study indicate, in conscious freely-voiding rats, that: (1) acute intravenous administration of moxonidine and clonidine evokes diuresis, natriuresis, kaliuresis, and urinary cGMP excretion that are inhibited by

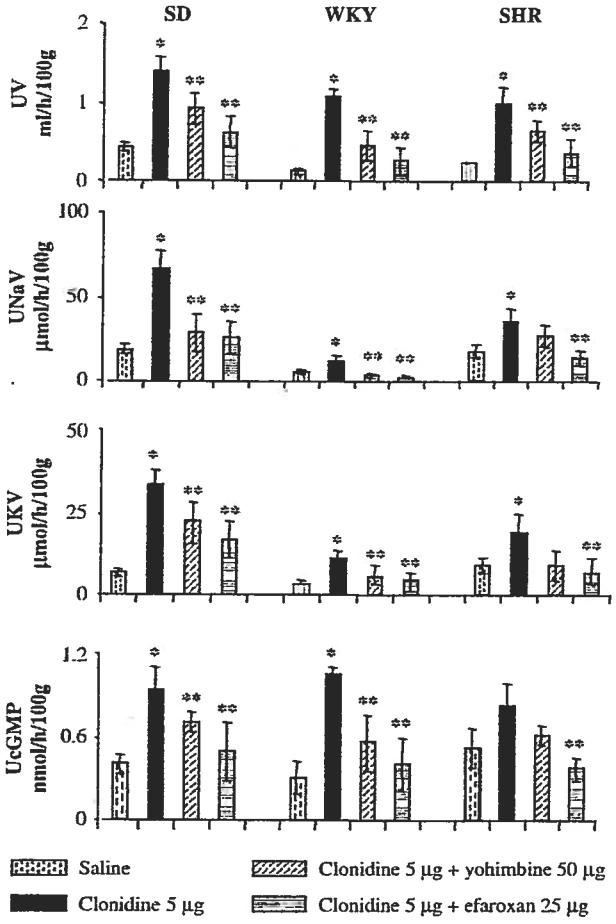

Figure 3 Urine output, sodium, potassium, and cGMP excretions during the first hour of treatment with clonidine with and without pretreatment with yohimbine and efaroxan in SD, WKY, and SHR ($n=8$–26 rats per group per treatment). *$P<0.001$ vs corresponding saline control. **$P<0.01$ vs corresponding clonidine.

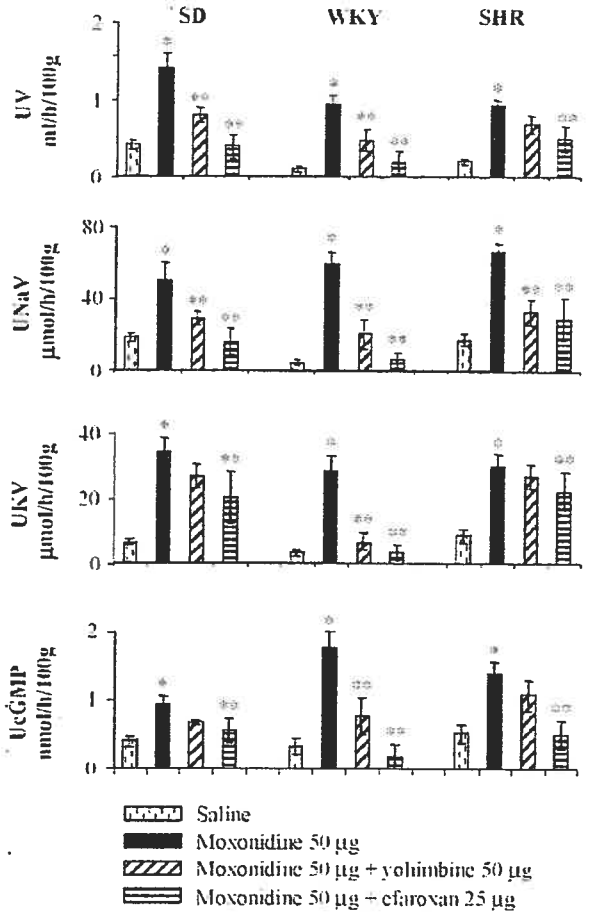

Figure 4 Urine output, sodium, potassium, and cGMP excretions during the first hour of treatment with moxonidine with and without pretreatment with yohimbine and efaroxan in SD, WKY, and SHR ($n=8$–26 rats per group per treatment). *$P<0.001$ vs corresponding saline control. **$P<0.01$ vs corresponding moxonidine.

efaroxan and yohimbine, implicating both I_1-receptors and α_2-adrenoceptors in the renal effects of these drugs, and making distinction between the contributions of either receptor not easy. (2) Regardless of the receptor type, the urinary effects of moxonidine are not consistently different in SHR from WKY and SD normotensive controls, and are, therefore, not influenced by hypertension *per se*. (3) Renal responses to moxonidine are associated with elevated plasma ANP, and are (4) dose-dependently inhibited by the natriuretic peptide antagonist. These studies show, for the first time, that natriuretic peptides mediate the renal effects of acute moxonidine treatment.

Several groups have reported that acute intravenous injections of moxonidine in normotensive and hypertensive rats evoke diuresis and natriuresis (Allan *et al*., 1993; Hohage *et al*., 1997a, b; Mukaddam-Daher & Gutkowska, 1999; 2000). These actions may be mediated centrally and peripherally (Smyth & Penner, 1998). Intravenous moxonidine crosses the blood–brain barrier to act preferentially on imidazoline receptors in the brainstem rostroventrolateral medulla (RVLM) (Haxiu *et al*., 1994), although an effect on α_2-adrenoceptors cannot be ruled out. Activation of both receptor types inhibits central sympathetic output to the peripheral vasculature, the heart, and kidneys. Inhibition of renal sympathetic nerve activity leads to diuresis and natriuresis, by modulating renin release, sodium reabsorption, or renal hemodynamics (Dibona, 2002). Activation of α_2-adrenoceptors in the RVLM can promote urinary sodium excretion by a renal nerve-dependent mechanism and increase the urine flow rate by a pathway that involves vasopressin secretion from the paraventricular nucleus (PVN) of the hypothalamus (Menegaz *et al*., 2001). Activation of α_2-adrenoceptors in micturition centres of the lumbosacral and supraspinal regions leads to bladder hyperactivity (Kontani *et al*., 2000). Imidazolines may also directly act on imidazoline receptors and/or α_2-adrenoceptors present in the kidney cortex and outer medulla (Bidet *et al*., 1990; Limon *et al*., 1992; Li & Smyth 1993a; Greven & von Bronewski-Schwarzer, 2001). Selective activation of renal I_1-receptors by intrarenal infusion of moxonidine markedly increases the urine flow rate and sodium excretion (Allan *et al*., 1993) by a direct tubular effect (Greven & von Bronewski-Schwarzer, 2001). Although moxonidine binds to renal I_1-receptors with higher affinity than to α_2-adrenoceptors, activation of α_2-adrenoceptors may inhibit vasopressin-dependent

(Edwards *et al.*, 1992; Nielsen *et al.*, 2002) and vasopressin-independent (Junaid *et al.*, 1999) aquaporin-mediated water reabsorption, or stimulate local nitric oxide release in the renal medulla (Zou & Cowley, 2000).

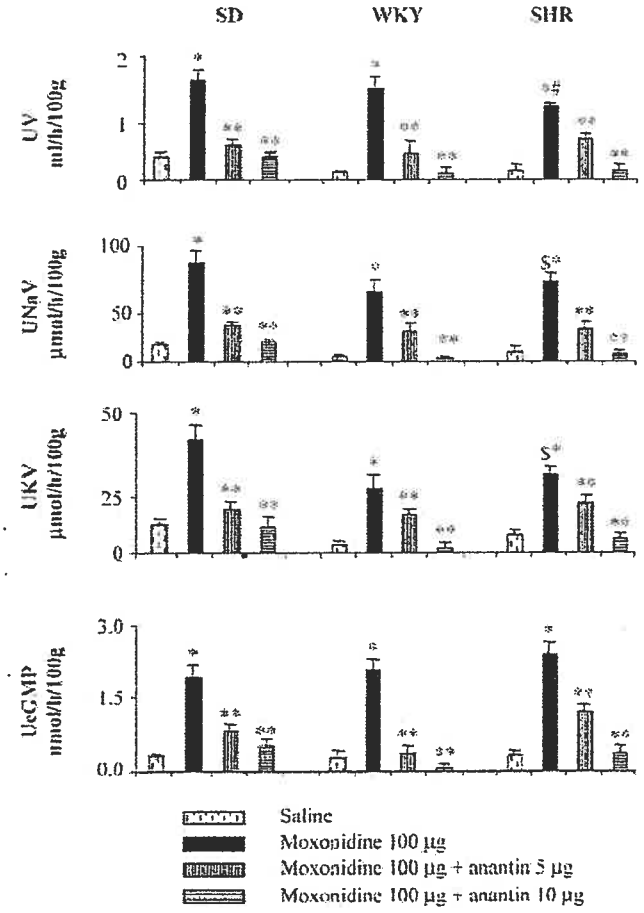

Figure 5 Urine output, sodium, potassium, and cGMP excretions during the first hour of treatment with anantin, administered 10 min before 100 µg moxonidine injection in SD, WKY, and SHR ($n=5$–26 rats per group per treatment). $^{*}P<0.001$ vs corresponding saline control. $^{**}P<0.001$ vs corresponding moxonidine; $^{\#}P<0.05$ vs corresponding SD and WKY; $^{\$}P<0.05$ vs corresponding SD.

Alternatively, Smyth *et al.* (2003) recently reported that low-dose moxonidine infused directly into the left renal artery resulted in similar levels of urine and sodium excretion from the left and the right kidneys, and accordingly suggested that an extra-renal diuretic and natriuretic factor mediated its renal effects. The present study shows that acute intravenous moxonidine in conscious rats increases plasma natriuretic peptides and urinary cGMP excretion. These renal effects are inhibited by anantin, a natriuretic peptide antagonist, providing clear evidence that the extra-renal factors, proposed by Smyth *et al.* (2003), are natriuretic peptides. The present findings substantiate the hypothesis that, regardless of the receptor type involved, intravenous moxonidine injections result in elevated levels of circulating natriuretic peptides, which would act on their receptors to stimulate diuresis and natriuresis.

Natriuretic peptides participate in cardiovascular regulation through direct vasodilating and renal effects, as well as by influencing the sympathetic nerve activity and heart rate (Jamison *et al.*, 1992; Imaizumi & Takeshita, 1993; Melo *et al.*, 2000; de Bold *et al.*, 2001). Intravenous administration of ANP in rats results in suppression of efferent activity in adrenal, renal, and splenic sympathetic nerve fibers, and the effect is absent in decerebrated rats, indicating that circulating ANP modulates autonomic outflows through hypothalamic neurons that lack a blood–brain barrier (Niijima, 1989). Also, circulating natriuretic peptides act on the kidney to cause diuresis and natriuresis by stimulating the glomerular filtration rate and renal blood flow, exerting direct actions on renal proximal tubules and inner medullary collecting duct cells to inhibit sodium and water reabsorption, and by inhibiting renin and vasopressin release and aldosterone synthesis and secretion (Jamison *et al.*, 1992; Imaizumi & Takeshita, 1993; Melo *et al.*, 2000; de Bold *et al.*, 2001).

In the present study, antagonism of natriuretic peptides resulted in complete inhibition of moxonidine-stimulated urinary parameters. Although three NPR subtypes (NPR-A, NPR-B, and NPR-C) are present in the kidney (Jamison *et al.*, 1992), NPR-A (also known as GC-A) was shown to be the receptor subtype that mediates the acute diuretic and natriuretic effects of the cardiac natriuretic peptides ANP and brain natriuretic peptide (BNP). In NPR-A knockout mice, rapid volume expansion, a primary stimulus of natriuretic peptides (ANP and BNP) release, fails to stimulate

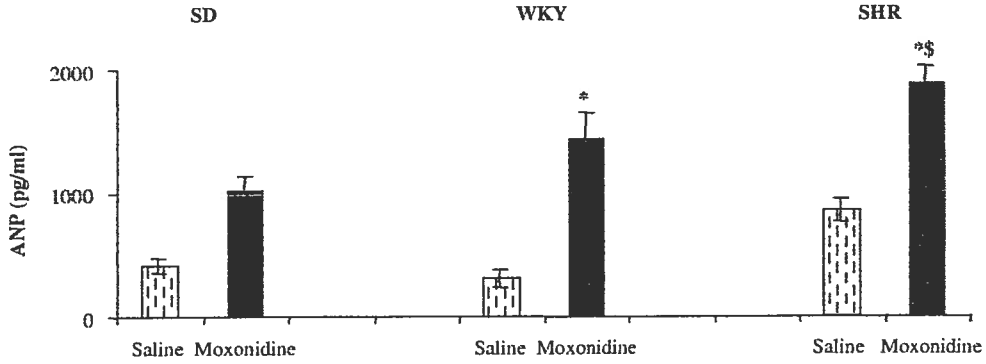

Figure 6 Effect of moxonidine on plasma ANP levels 15–20 min after moxonidine injection in SD, WKY, and SHR ($n=5$–18 rats per group per treatment). $^{*}P<0.01$ vs corresponding saline control; $^{\$}P<0.001$ vs corresponding SD.

water and sodium excretion (Kishimoto *et al.*, 1996). Therefore, we may propose that natriuretic peptides mediate the effects of moxonidine, most likely through their NPR-A. In fact, it would be interesting, at this point, to study the effects of moxonidine in NPR-A null mice.

Investigating whether the renal effects of moxonidine are altered in hypertension, the present experiments demonstrate that the responses in SHR were not consistently different from those in two normotensive control strains. Intriguingly, differences were more influenced by strain than pressure. Moxonidine-stimulated diuresis was lower in SHR compared to normotensive SD and WKY rats. On the other hand, natriuresis and kaliuresis were lower in SHR than in SD but not in WKY rats. The lack of stimulated renal effects by moxonidine in SHR is contrary to the expectation that inhibition of hypertension-associated renal sympathetic overactivity, which tends to promote sodium and water retention to a greater extent than in normotensive rats (Roman & Cowley, 1985), may result in enhanced renal responses to moxonidine. Furthermore, plasma natriuretic peptide levels, which were already higher in SHR than in WKY and SD rats, were further elevated by moxonidine, but the increase in circulating levels was not reflected by the urinary parameters. This is not surprising, however, because diuresis and natriuresis are the net product of multiple hemodynamic, neural, hormonal, and local factors that may be altered in hypertension (Li & Smyth, 1993b; Dibona, 2002), including renal I_1-receptors, α_2-adrenoceptors, and NPRs. Previous studies have shown that the density of renal α_2-adrenoceptors is elevated in SHR (Stanko & Smyth, 1991), but not in 1K1C hypertensive rats (Li & Smyth, 1993a). Idazoxan-labelled imidazoline receptor binding is lower in 1K1C hypertensive rat kidneys compared to sham controls (Li & Smyth, 1993a). On the other hand, renal NPRs in SHR are upregulated in the inner medulla (Guillaume *et al.*, 1997), and are either unchanged (Tremblay *et al.*, 1993) or reduced in glomeruli (Guillaume *et al.*, 1997). Therefore, renal receptor regulation may counter-balance hormonal levels.

The reduced renal responses to moxonidine in SHR may also be explained by a greater drop in blood pressure in SHR following treatment. We have shown in previous studies that injections of 50 μg moxonidine in normotensive SD rats do not significantly reduce blood pressures (diastolic, systolic and mean), measured by radiotelemetry (Mukaddam-Daher & Gutkowska, 2000). Also, 50 μg moxonidine, which only slightly decreased systolic blood pressure measured by the tail-cuff method 30 min after injection in WKY rats ($\sim$10 mmHg), resulted in a significant ($\sim$40 mmHg) decrease in SHR (Mukaddam-Daher & Gutkowska, 1999). However, although the drop in blood pressure may explain, in part, the diuretic effect of moxonidine, it may not explain the natriuretic effect, which was not different between SHR and WKY.

Most importantly, whereas characterization of the receptor type mediating the renal effects of moxonidine in these conditions is not conclusive, this study proves, for the first time, that natriuretic peptides are directly involved in the renal actions of acute intravenous moxonidine in conscious rats, and that its actions are not altered by hypertension.

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Chronic Imidazoline Receptor Activation in Spontaneously Hypertensive Rats

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Background: Acute intravenous administration of moxonidine, an imidazoline I_1-receptor agonist, reduces blood pressure (BP) in normotensive and hypertensive rats, induces diuresis and natriuresis, and stimulates plasma atrial natriuretic peptide (ANP). In these studies we investigated the involvement of natriuretic peptides (ANP and brain natriuretic peptide) in the effects of chronic activation of imidazoline receptors.

Methods: Spontaneously hypertensive rats (SHR; 12 to 14 weeks old) received 7-day moxonidine treatment at various doses (10, 20, 60, and 120 μg/kg/h) via subcutaneously implanted osmotic minipumps.

Results: Hemodynamic parameters (continuously monitored by telemetry) revealed that, compared with saline-treated rats, moxonidine dose-dependently decreased blood pressures (BPs). Maximal blood pressure lowering effect was achieved by day 4 of treatment, at which point 60 μg/kg/h reduced mean arterial pressure (MAP) by 14.5 $\pm$ 6.8 mm Hg as compared with basal levels. The decrease in MAP was influenced by a drop in both diastolic and systolic pressures. Moxonidine treatment did not alter daily urinary sodium and potassium excretions, but 120 μg/kg/h moxonidine decreased urine volume after 2 days and increased cyclic guanosine 3′5′monophosphate excretion on days 4 to 7 of treatment. Chronic moxonidine treatment dose-dependently increased plasma ANP to reach, at 120 μg/kg/h, a 40% increase ($P < .01$) above that of corresponding saline-treated SHR, with a concomitant increase in left and right atrial ANP mRNA (more than twofold). Plasma BNP increased by 120 μg/kg/h moxonidine (11.0 $\pm$ 1.1 *v* 16.5 $\pm$ 1.9 pg/mL, $P < .002$) without significant increases in atrial and ventricular BNP mRNA.

Conclusions: ANP and BNP may be involved in the antihypertensive effect of chronic moxonidine treatment. Accordingly, natriuretic peptides may contribute to the sympatholytic and cardioprotective effects of chronic activation of imidazoline I_1-receptors. Am J Hypertens 2002;15:803–808 © 2002 American Journal of Hypertension, Ltd.

Key Words: Moxonidine, ANP, BNP, blood pressure, osmotic minipumps, telemetry, LVH, natriuresis.

Activation of the sympathetic outflow to the heart, kidneys, and skeletal muscle vasculature is commonly present in young (<45 years) patients with essential hypertension. The sympathetic stimulation leads to cardiac risks such as development of left ventricular hypertrophy (LVH), predisposing to ventricular arrythmias, increasing insulin resistance, and accelerating atherogenesis. Consequences of increased sympathetic activity and elevated levels of catecholamines in essential hypertension can be minimized by reduction of sympathetic outflow by centrally acting antihypertensive agents. However, despite their efficacy, the use of these drugs has been often limited by their adverse effects, such as dry mouth and sedation. Recently, newly developed, centrally acting antihypertensive drugs (ie., moxonidine and rilmenidine) that have fewer adverse effects and show high affinity for the nonadrenergic imidazoline I_1-receptors and low affinity for α_2-adrenergic receptors, have been introduced in hypertension treatment. These drugs reduce blood pressure (BP) by selective activation of I_1-receptors in the central nervous system,[1,2] inducing an inhibition of sympathetic tone,[3,4] and by direct actions on imidazoline receptors in the kidney to cause diuresis and natriuresis,[5,6] both mechanisms leading to acute and long-term control of pressure.

Studies from this laboratory have shown that acute

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intravenous moxonidine administration to normotensive[7] and spontaneously hypertensive rats[8] reduces BP, increases plasma atrial natriuretic peptide (ANP), and stimulates urine flow and the excretion of sodium, potassium, and cyclic guanosine 3′5′ monophosphate (cGMP), the index of ANP activity, indicating that ANP is involved in the acute effects of moxonidine.

The natriuretic peptides, ANP and brain natriuretic peptide (BNP), primarily produced by the cardiac atria and ventricles, have potent diuretic, natriuretic, and vasorelaxant activities. Natriuretic peptides inhibit cell proliferation and extracellular matrix production of cardiac fibroblasts through inhibition of the renin-angiotensin and endothelin systems.[9] The actions of these peptides are mediated by a common guanylyl cyclase coupled natriuretic peptide receptor (NPR-A), leading to increased intracellular cGMP production, and another clearance (NPR-C) receptor through inhibition of adenylyl cyclase/cyclic adenosine 3′5′ monophosphate (cAMP).

Because of their established role in volume and pressure regulation, natriuretic peptides may contribute to the antihypertensive effects of chronic activation of imidazoline receptors by moxonidine. Therefore, the cardiovascular and renal effects of chronic moxonidine therapy and the possible involvement of natriuretic peptides in these effects were investigated in conscious spontaneously hypertensive rats (SHR), a model of human essential hypertension.

Methods

Female spontaneously hypertensive rats (SHR; 12 to 14 weeks old) and normotensive Sprague-Dawley rats (SD; 200 to 225 g) were purchased from Charles River (St. Constant, Quebec, Canada). The animals were housed at 22°C, maintained on a 12-h light/12-h dark cycle, and fed Purina Rat Chow (Ralston Purina) and tap water ad libitum for at least 3 days before experimentation. All procedures were carried out with the approval of the Bioethics Committee of CHUM, according to the Canadian Guidelines.

Hemodynamic Measurements

Blood pressures, systolic, diastolic, and mean arterial pressure (MAP), heart rate, and locomotor activity in freely moving rats were measured by telemetry (Data Sciences International, St. Paul, MN), as we have previously described.[7,10] The animals were anesthetized with pentobarbital, and the flexible transmitter catheter was secured surgically in the abdominal aorta below the renal arteries pointing against the flow. The transmitter was sutured to the abdominal wall. The rats were placed separately in cages, and each cage was placed over the receiver panel and connected to the computer for collection of data. The monitoring system consisted of a pressure transmitter (radio frequency transducer model TL11M2-C50-PXT), receiver panel, consolidation matrix, and personal computer with program. Output from the transmitter was monitored by the receiver (RLA 2000). The signals from the receiver were consolidated by the multiplexer (BCM 100) and were stored and analyzed by microcomputer with software (A.R.T., Dataquest, Data Sciences International). The pressure signals were corrected automatically for changes in atmospheric pressure.

The animals were allowed to recover for at least 10 days before experimentation. Then, under isoflurane anesthesia, animals were randomly implanted subcutaneously with osmotic minipumps (2ML1, Alzet Corp., Cupertino, CA) that allowed continuous delivery of moxonidine (10, 20, and 60 μg/kg/h) or vehicle at the rate of 10 μL/h, for 1 week. Rats were placed in their cages over corresponding pressure receivers. Data from all parameters were collected every min over 24 h before and for 7 days during moxonidine or vehicle treatment.

Moxonidine (generous gift of Solvay Pharmaceuticals, GmbH, Hannover, Germany) solution was prepared by dissolving the drug in isotonic saline, acidified with 1 N HCL (pH < 6.5), then adjusted to pH 7.0 to 7.4 with 1 N NaOH.

Renal Effects of Moxonidine

In other groups of normotensive and hypertensive rats, the renal responses to various moxonidine treatments were investigated by assessment of diuresis, natriuresis, and kaliuresis as well as urinary cGMP (UcGMP) excretion. Animals were placed in metabolic cages for 3 days before experimentation. They were then randomly separated into four groups and implanted with osmotic minipumps (as described above), containing either moxonidine (10, 60, or 120 μg/kg/h) or saline vehicle. The rats were again placed in their metabolic cages. Body weight, water and food intake, and 24-h urine output were measured daily at 8:30 AM, 2 days before and during 7 days of moxonidine or vehicle treatment.

Sodium and potassium concentrations were measured in urine with a flame photometer (Instrumentation Laboratory, Lexington, MA). Urinary cGMP was measured in serial dilutions by a specific radioimmunoassay as previously described.[10]

Plasma and Tissue Natriuretic Peptide Measurement

The animals were killed by decapitation on day 7 of moxonidine treatment. Trunk blood (1 mL) was collected in prechilled tubes containing protease inhibitors in a final concentration: 10 μmol/L EDTA, 10 μmol/L phenylmethylsulfonyl fluoride (PMSF), and 5 μmol/L pepstatin A (Sigma Chemical Co. St. Louis, MO). After blood centrifugation at 4°C, plasma was collected and stored at −80°C. The hearts were rapidly excised. The atria and ventricles were dissected and frozen in liquid nitrogen and then stored at −80°C.

For measurement of natriuretic peptide content, the tissues were thawed in 0.1 mol/L acetic acid containing

protease inhibitors (as described above) at 4°C. The tissues were homogenized twice in a Polytron homogenizer and centrifuged at 30,000 *g* for 20 min at 4°C. The retrieved supernatants were combined and stored at −80°C.

The ANP and BNP were measured by radioimmunoassay[11,12] in plasma after extraction by Sep-Pak C18 cartridges (Millipore, Mississauga, ON, Canada) and in serial dilutions of tissue homogenates using specific antibodies. Rat ANP_{1-28} and BNP-32 were iodinated with ^{125}I-Na using lactoperoxydase and purified by high performance liquid chromatography. Proteins were measured spectrophotometrically (absorbance 595 nmol/L), using bovine serum albumin (BSA) as standard.

Total RNA Extraction and Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

The ANP and BNP gene expression in the heart chambers were detected by RT-PCR as we previously described.[12] Total RNA was isolated from the rat hearts using TRIZOL reagent (Life Technologies, Inc., Burlington, ON, Canada) according to the manufacturer's specifications and treated with RNase-free DNase I under a standard protocol. The integrity and quality of the purified RNA were controlled by formaldehyde denaturing agarose gel electrophoresis and by measurement of the A260/A280 nm ratio. First strand cDNA was synthesized in a final volume of 40 μL containing first strand buffer, 2 μg rat cardiac RNA used as a control, 2 μg hexanucleotide primer (Pharmacia, Mississauga, ON, Canada), and avian myeloblastosis virus (AMV) reverse transcriptase (12 units/μg RNA; Life Technologies, Inc.). For PCR amplification, the forward ANP primer spanned the junction of exon 1 and 2 of ANP gene (bp170 to 198), and reverse (bp 527 to 494) spanned the junction of exon 2 and 3 of ANP DNA. The forward BNP primer (bp −36 to −4) spanned sequences in the 5′-untranslated region on the first exon and reverse primer spanned translated (bp 334 to 316) sequence on the second exon of rat BNP gene. A quantity of 10 μL of the PCR products was electrophoresed on 1.5 agarose gel in the presence of ethidium bromide. Fluorescent bands were counted and analyzed with the Storm 840 Imaging System and ImageQuant software (version 5.1, Molecular Dynamics, Sunnyvale, CA). To validate the use of this RT-PCR assay as a tool for the semiquantitative measurement of ANP mRNA and BNP mRNA, dose-response curves were established for different amounts of total RNA, and the samples were quantified in the curvilinear phase of PCR amplification. These data were normalized to the corresponding values of 18S RNA PCR products in the same samples (amplified by RT-PCR with primers manufactured by Ambion Inc., Austin, TX).

Statistical Analysis

Telemetric data obtained each 1-min from each rat over 24 h were pooled. All parameter values were expressed as difference from baseline. Comparisons between groups were made by analysis of variance (ANOVA). The paired Student *t* test was used in each group to compare initial to final values, and the nonpaired *t* test to compare values in moxonidine treated rats versus corresponding saline-treated controls. $P < .05$ was considered significant. All data are expressed as means ± SEM.

Results

Compared to saline-treatment, 1-week treatment with moxonidine had no significant effect on water and food intake or on body weight. However, to rule out any effect of body weight difference among the various study groups, all renal parameters were normalized to percent body weight.

Compared with basal, urine volume decreased on day 1 of saline or 10 and 60 μg/kg/h moxonidine treatment, but attained balance by day 4. The dose of 120 μg/kg/h reduced urine excretion from a basal of 14.3 ± 0.6 to 11.2 ± 0.9 mL ($P < .05$) on day 7. On the other hand, cGMP increased (160%, $P < .04$) by 120 μg/kg/h moxonidine starting on day 4 but did not significantly change thereafter. Urinary sodium and potassium concentrations significantly increased by treatment as compared with basal values. However, the increase disappeared when daily excretions were calculated. Serum creatinine levels were not altered by either treatment, indicating maintained renal function.

During the first 2 days of treatment, all groups, including saline-treated controls showed a transient decrease from basal values in activity pattern, but returned to normal for the remaining period of treatment.

Basal pressure measurements were not different among the various hypertensive groups. Basal systolic pressure in all SHR was 170.3 ± 4.2 mm Hg, diastolic pressure, 118.4 ± 3.1 mm Hg, and MAP 143.4 ± 3.6 mm Hg. Fig. 1 shows that, compared with basal values, treatment with 10, 20, and 60 μg/kg/h moxonidine resulted in a dose-dependent reduction in systolic, diastolic, and mean arterial pressures. Maximal BP lowering effect was achieved by day 4 of treatment; but, thereafter, treatment did not cause any further reduction in pressures. Moxonidine at 60 μg/kg/h reduced MAP on day 4 by 14.5 ± 6.8 mm Hg from basal. The decrease in MAP was influenced by a drop in both diastolic and systolic pressures (Fig. 1).

Baseline HR in SHR was 349 ± 7 beats/min. Fig. 1 shows that there were no significant bradycardic effects of various doses of moxonidine in spontaneously hypertensive rats.

The hypertensive rats showed a higher left ventricular weight to body weight (LV/BW) ratio than normotensive rats (2.32 ± 0.08 *v* 3.06 ± 0.05 mg/g, $P < .001$), indicating left ventricular hypertrophy in SHR 12 to 14 weeks-old. The LV/BW ratio was not altered by 7-day treatment with 120 μg/kg/h of moxonidine in normotensive and hypertensive rats. However, treatment resulted in a significant decrease in left ventricular weight in hypertensive rats (585.30 ± 0.01 *v* 542.40 ± 0.01 mg, $P < .004$).

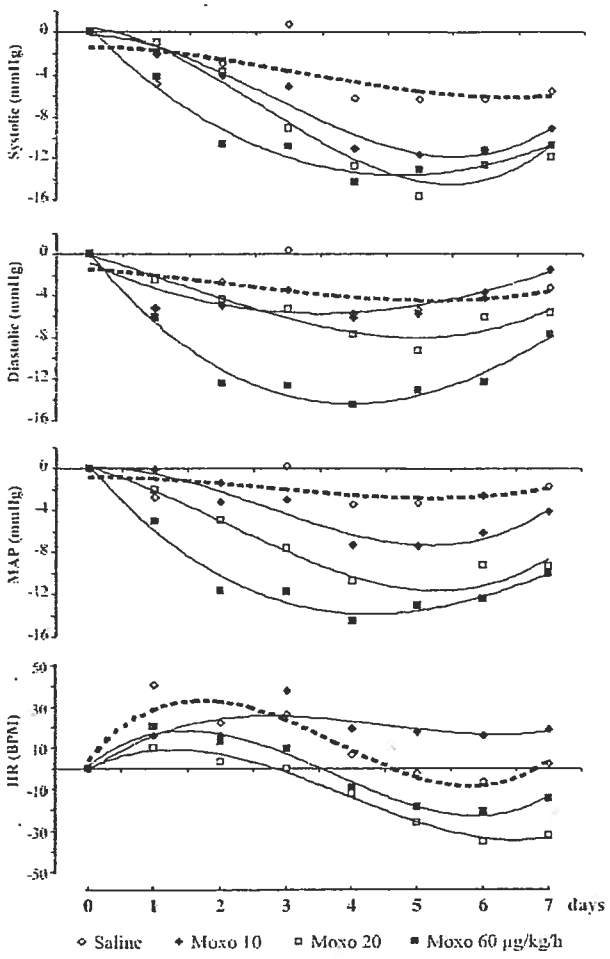

FIG. 1. Effect of 7-day treatment of spontaneously hypertensive rats with different doses of moxonidine (10, 20, and 60 µg/kg/h) on hemodynamic parameters (n = 2 to 6 rats/group), represented as difference from basal. MAP = mean arterial pressure; HR = heart rate (beats/min); Moxo = moxonidine.

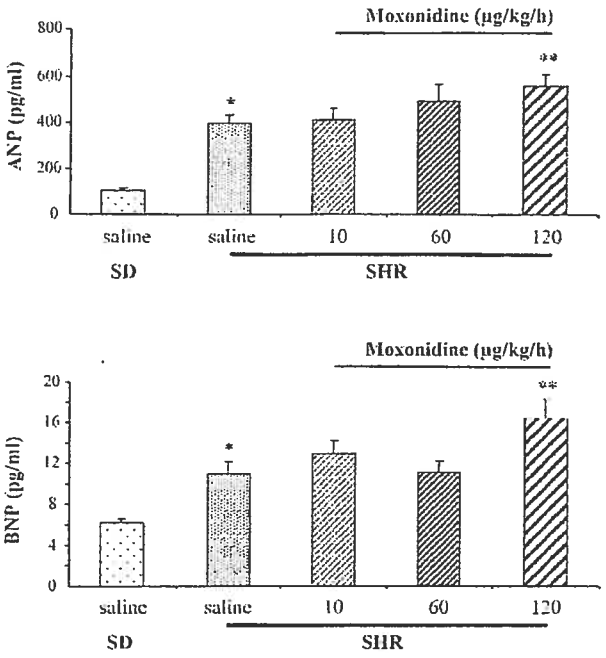

FIG. 2. Dose-dependent effect of 7-day moxonidine treatment (10, 60 and 120 µg/kg/h) on plasma atrial natriuretic peptide (ANP) and brain natriuretic peptide (BNP) in spontaneously hypertensive rats (SHR). *P < .02 $v.$ Sprague-Dawley (SD) normotensive control rats receiving saline vehicle; **P < .02 $v.$ saline-treated SHR; n = 7 to 20 rats/group.

Basal plasma ANP levels were significantly higher in SHR than in normotensive rats (104 ± 10 v 398 ± 32 pg/mL, P < .001). Chronic treatment of SHR with moxonidine (10, 60, and 120 µg/kg/h) induced further dose-dependent increases in plasma ANP and represented, with the highest dose of 120 µg/kg/h, a 40% increase (P < .01) above corresponding saline-treated hypertensive controls (Fig. 2).

Plasma BNP was also significantly higher in SHR than in normotensive rats (6.2 ± 0.4 v 11.0 ± 1.1 pg/mL, P < .001). Moxonidine dose-dependently stimulated plasma BNP, so that it represented 16.5 ± 1.9 pg/mL (P < .002) at 120 µg/kg/h (Fig. 2).

Tissue ANP and BNP content was measured in atria and ventricles from normotensive SD rats and hypertensive SHR treated with saline-vehicle or 120 µg/kg/h moxonidine for 7 days. The ANP was lower in both ventricles and in the left, but not the right, atria of SHR as compared with normotensive rats. However, after chronic treatment of SHR with moxonidine, ANP concentrations decreased significantly in the left (P < .01) and right (P < .001) atria (Fig. 3), and remained unchanged in the ventricles.

Hypertension decreased BNP content in the left but not the right atria. Treatment with moxonidine resulted in a further decrease in left atrial BNP content (6.0 ± 0.5 $v.$ 2.7 ± 0.2 ng/mg protein, P < .03), without affecting right atrial or ventricular levels.

The ANP and BNP mRNA were measured in the atria and ventricles from SHR treated with moxonidine (10, 60, and 120 µg/kg/h) for 7 days. Moxonidine treatment in-

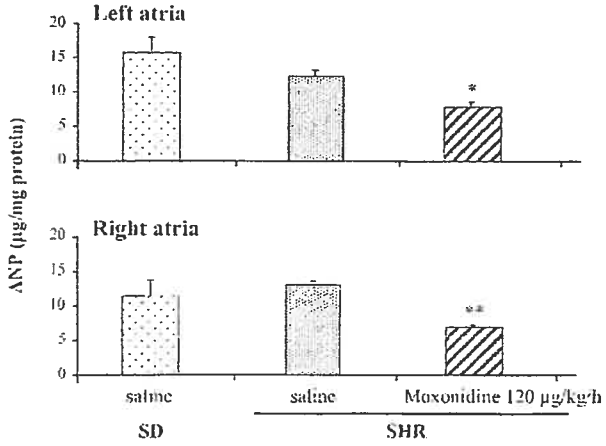

FIG. 3. Effect of 7-day moxonidine treatment (120 µg/kg/h) on left and right atrial ANP content in SHR. *P < .01, **P < .001 $v.$ saline-treated SHR; n = 4 rats/group. Abbreviations as in Fig. 2.

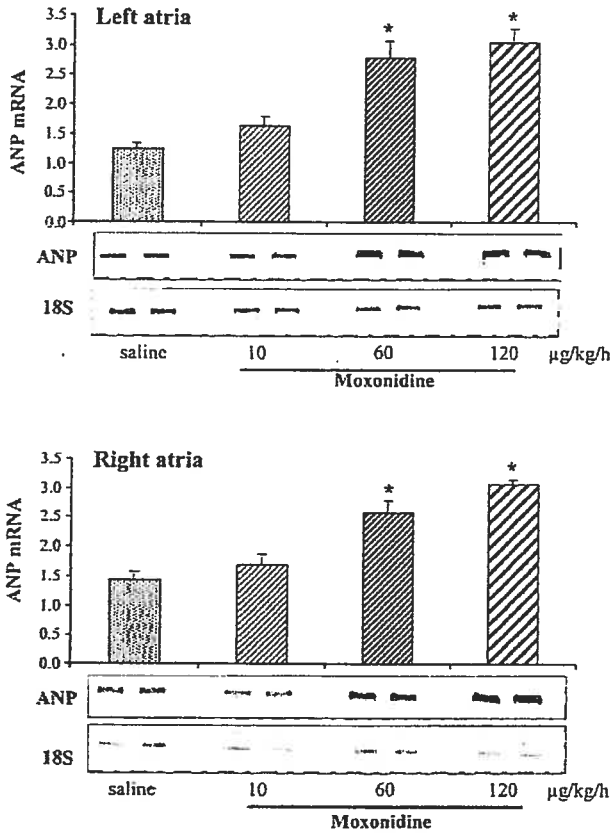

FIG. 4. Dose-dependent effect of chronic moxonidine treatment (10, 60, and 120 μg/kg/h) on left and right atrial ANP mRNA in SHR as normalized to 18S mRNA. *$P < .001$ *v.* saline-treated SHR; $n = 6$ rats/group. Abbreviations as in Figs. 2 and 3.

duced significant and dose-dependent increases in left and right atrial ANP mRNA when compared with those of saline-treated rats. Fig. 4 shows that 120 μg/kg/h moxonidine increased ANP mRNA by 2.1- and 2.4-fold in the right and left atria, respectively. Moxonidine also increased ANP mRNA by 26% in left ventricles (data not shown). On the other hand, moxonidine did not change BNP mRNA in SHR left and right atria, but resulted in a 24% increase in the left ventricles.

Discussion

The present study shows that chronic treatment of hypertensive rats with moxonidine decreases blood pressure at doses that have minimal effects on animal activity, indicating reduced or absence of sedation, which is normally observed with α_2-adrenergic agonists.[13–15] The decrease in pressure is associated with increased excretion of urinary cGMP as well as increased atrial synthesis and release of natriuretic peptides, ANP, and BNP. These findings support the notion that, in addition to activation of imidazoline receptors in the brain and kidneys, the chronic antihypertensive action of moxonidine may include stimulation of natriuretic peptides, which would contribute to the maintenance of BP reduction and cardioprotection by imidazoline compounds.

Selective activation of central imidazoline I_1-receptors, located in the rostral ventro-lateral medulla (RVLM), results in inhibition of peripheral sympathetic activity and produces arterial vasodilation.[1–5] In the present study, chronic activation of imidazoline I_1-receptors by moxonidine at various doses attenuated the high BP, starting by the first day of treatment, and lower blood pressure was maintained throughout the 7 days of the study. The magnitude of reduction in systolic and diastolic BPs was within the range previously reported by others in SHR treated orally with moxonidine at 8 to 10 mg/kg/day.[16,17]

Stimulated natriuretic peptides would contribute to the pressure lowering effect of moxonidine through inhibiting sympathetic activity and decreasing total peripheral resistance.[18,19] The sympatholytic effect of ANP has been shown in transgenic mice in which a 20-fold increase in plasma ANP was associated with a reduction of 20 mm Hg in blood pressure.[20] In contrast, mice with a homozygous disruption of the Pro-ANP gene (−/−) that fail to synthesize ANP developed chronic hypertension due to elevated total peripheral resistance, as determined by an increase in cardiovascular autonomic tone.[21] Similarly, transgenic mice over-expressing BNP exhibit reduced BP that is accompanied by an elevation of plasma cGMP concentrations, and BNP-transgenic mice lacking natriuretic peptide receptor A (NPR-A) are hypertensive.[22] Therefore, it is reasonable to suggest that moxonidine-stimulated ANP and BNP may contribute to attenuation of sympathetic tone by moxonidine.

The BP lowering effect of centrally acting imidazoline receptor agonists is associated with inhibition of renal sympathetic nerve activity, and with subsequent diuresis and natriuresis.[6] These enhanced renal responses have been observed after acute,[7,8] but not chronic, moxonidine treatment.[23] It is important to note that imidazoline antihypertensive agents are not diuretics. However, unlike other sympatholytic agents, imidazoline compounds do not cause sodium retention,[24] suggesting the presence of intrarenal mechanisms counteracting sodium-retaining effects that result from sympatho-inhibition and lower blood pressure. In support of this presumption, a shift in the pressure natriuresis curve was observed in rats after rilmenidine (selective I_1-receptor agonist) treatment, indicating a significant contribution of renal mechanisms to long-term BP control by I_1-receptor agonists.[25] Renal mechanisms that could contribute to the maintenance of sodium balance may include direct renal action of the drug. In fact, I_1-imidazoline receptors have been found in the kidney,[1] and direct infusion of moxonidine into the renal artery increases sodium and water excretion.[6,26] In addition, treatment-stimulated natriuretic peptides would act on their own receptors in the kidney to maintain sodium balance.

Previous studies have shown that SHR develop LVH at 4 weeks of age, even before blood pressure starts to increase. In the present study, SHR 12 to 14 weeks old with established hypertension showed elevated left ven-

tricular to body weight ratio, confirming development of left ventricular hypertrophy. Although relatively short, the 7-day treatment decreased left ventricular weight, implying that treatment of a longer duration may have further beneficial effects. The reversal of left ventricular hypertrophy may be influenced by stimulated natriuretic peptides. Independent of their role in BP control, natriuretic peptides have direct antihypertrophic and antifibrotic actions on the heart.[27] The NPR-A system has intrinsic growth inhibitory properties in noncardiac and cardiac cells in vitro,[28] and in endothelial and vascular smooth muscle cells, ANP is antimitogenic.[29] Mice lacking NPR-A display a marked cardiac hypertrophy and chamber dilation by 3 months of age,[30] and mice with disrupted BNP develop multifocal fibrotic lesions in the ventricles.[31]

Taken together, these data show that chronic activation of imidazoline receptors in hypertensive rats is associated with stimulated production and synthesis of natriuretic peptides, which may contribute to the long-term sympatholytic and cardioprotective effects of the treatment.

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Imidazoline Receptors in the Heart: Characterization, Distribution, and Regulation

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Summary: Imidazoline receptors were identified in cardiac tissues of various species. Imidazoline receptors were immunolocalized in the rat heart. Membrane binding and autoradiography on frozen heart sections using 0.5 n*M* para-iodoclonidine (^{125}I-PIC) revealed that binding was equally and concentration-dependently inhibited by epinephrine and imidazole-4-acetic acid (IAA), implying ^{125}I-PIC binding to cardiac α2-adrenergic and I1-receptors, respectively. After irreversible blockade of α2-adrenergic receptors, binding was inhibited by the selective I1-agonist, moxonidine, and the I1-antagonist, efaroxan, in a concentration-dependent (10^{-12} to 10^{-5} *M*) manner. Calculation of kinetic parameters revealed that in canine left and right atria, I1-receptor Bmax was 13.4 ± 1.7 and 20.1 ± 3.0 fmol/mg protein, respectively. Compared to age-matched normotensive Wistar Kyoto rats, I1-receptors were increased in 12-week-old hypertensive rat (SHR) right (22.6 ± 0.3 to 43.7 ± 4.4 fmol/unit area, $p < 0.01$) and left atria (13.3 ± 0.6 to 30.2 ± 4.1 fmol/unit area, $p < 0.01$). Also, compared to corresponding normal controls, Bmax was increased in hearts of hamsters with advanced cardiomyopathy (13.9 ± 0.4 to. 26.0 ± 2.3 fmol/unit area, $p < 0.01$) and in human ventricles with heart failure (12.6 ± 1.3 to 35.5 ± 2.9 fmol/mg protein, $p < 0.003$). These studies demonstrate that the heart possesses imidazoline I1-receptors that are up-regulated in the presence of hypertension or heart failure, which would suggest their involvement in cardiovascular regulation. **Key Words:** α_2-Adrenergic receptors—Autoradiography—Heart failure—Hypertension—Immunolocalization—Membrane binding—Moxonidine—Paraiodoclonidine.

Imidazoline anti-hypertensive drugs that were originally thought to be α_2-adrenergic agonists also bind to nonadrenergic sites that have been named *imidazoline sites*. These sites are distinct from adrenergic and histaminergic sites as they show low affinity for the catecholamines epinephrine and norepinephrine as well as histamine (1–3). Imidazoline binding sites are now considered to be receptors because they fulfil the criteria for identification as receptors, including specificity of binding, association with physiologic function, and hav-

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ing endogenous ligands (4). Imidazoline receptor subtypes have been defined according to their ligand affinity. Binding sites named I_1 display higher affinity for ^{3}H-clonidine or clonidine analogues, whereas I_2 sites show a 100-fold lower affinity for clonidine but high affinity for ^{3}H-idazoxan (5,6). I_1 sites are present in brain stem, kidney, adrenal chromaffin cells, and carotid body (7–9) and have been directly associated with a regulation of vasomotor tone and the hypotensive mechanism of action of imidazoline drugs such as clonidine and moxonidine (1,10,11).

We have previously shown that in the normotensive conscious rat, activation of imidazoline receptors by acute IV administration of moxonidine, a highly selective imidazoline receptor agonist, is associated with increased plasma atrial natriuretic peptide (ANP) and urinary cyclic guanosine monophosphate, the index of ANP activity. The effects of moxonidine are inhibited completely by efaroxan, a selective imidazoline I_1 receptor antagonist, and partially by yohimbine, an α_2-adrenergic receptor antagonist (12). The magnitude of moxonidine effects was altered in hypertensive rats (13). Because the heart is the major site of ANP production, these studies led us to propose that imidazoline I_1 receptors are present in the heart and that they may be regulated in cardiovascular diseases. Therefore, studies were performed to identify, characterize, and localize I_1 receptors in the normal heart and to investigate possible regulation in hypertension and heart failure, two diseases characterized by stimulated sympathetic efferent neuronal activity.

METHODS

Cardiac receptors were identified in normotensive rats, hamsters, dogs, sheep, and humans. Receptor regulation was investigated in spontaneously hypertensive rats (SHRs, 12 weeks old), in comparison with age-matched normotensive Wistar-Kyoto (WKY) rats, and in normal Golden Syrian hamsters and hamsters with advanced cardiomyopathy (aged > 250 days). Rats and hamsters were purchased from Charles River (St. Constant, Quebec, Canada). Human ventricles (normal and with heart failure of unknown cause) were obtained from a tissue bank. Sheep and dog heart tissues were kindly supplied by other laboratories.

Experiments were performed according to the Canadian Guidelines. Animals were housed in a temperature- and light-controlled room with food and water ad libitum and maintained for at least 3 days before experimentation. After the animals were killed, hearts were excised, flash frozen in liquid nitrogen, and stored at −80°C.

Preparation of heart membranes

Heart membranes were prepared as previously described (14). In brief, dog, sheep, and human heart tissue specimens were homogenized with a Polytron homogenizer (Brinkmann Instruments, Westbury, NY) at 4°C in ice-cold homogenization buffer (50 m*M* tromethamine hydrochloride, pH 7.7; sucrose 10.27%; 5 m*M* ethylenediamine tetra-acetic acid [EDTA]; 5 m*M* egtazic acid; 100 μ*M* phenanthroline; and 50 μ*M* phenylmethylsulfonyl fluoride [PMSF]). After centrifugation at 2,000*g* for 5 min, 4°C, supernatants were collected and centrifuged at 30,000 *g*, 4°C for 35 min. Pellets were washed with tromethamine hydrochloride (50 m*M*, pH 7.7) containing 5 m*M* EDTA, then centrifuged. The pellets were resuspended in tromethamine hydrochloride buffer (50 m*M*, pH 7.7) and divided into two fractions: one fraction was aliquoted and frozen in liquid nitrogen and the other was incubated at room temperature for 35 min with ethylmaleimide (0.5 m*M*) (inactivates α_2-adrenergic binding) and phenoxybenzamine (1 μ*M*) (α-adrenergic alkylating agent), both prepared in tromethamine hydrochloride buffer, pH 7.7, as described by Ernsberger and Shen (11). The homogenates were centrifuged at 30,000 *g*, 4°C, for 35 min; then the pellets were washed with 50 m*M* tromethamine hydrochloride buffer (pH 7.7) and recentrifuged. The pellets were suspended in 50 m*M* tromethamine hydrochloride buffer (pH 7.7), aliquoted, then flash frozen in liquid nitrogen and stored at −80°C until use.

Protein was determined spectrophotometrically by a modification of Bradford (15) using bovine serum albumin as standard.

Membrane binding assays

Optimal binding conditions (amount of radiolabeled ligand, membrane protein concentration, and incubation time) were determined in preliminary studies according to Ernsberger and Shen (11). Membranes (200 μg) were incubated with ^{125}I-paraiodoclonidine (PIC) (100,000 cpm/50 μl) and 100 μl of drug or vehicle, in a total volume of 250 μl for 1 h at room temperature. The binding buffer consisted of 50 m*M* tromethamine hydrochloride, pH 7.7, 5 m*M* EDTA, 5 m*M* egtazic acid, 0.5 m*M* $MgCl_2$, and 50 μ*M* PMSF. Specificity of binding was determined by increasing concentrations (10^{-12}–10^{-5} *M*) of inhibiting drugs: moxonidine, (−)epinephrine, efaroxan, imidazole-4-acetic acid (IAA), prazosin, and rauwolscine. The reaction was stopped by the addition of ice-cold tromethamine hydrochloride binding buffer, and rapid vacuum filtration on glass microfibre (GF/C) (Whatman, Kent, UK) filters presoaked overnight in 0.1% polyethylenimine. The filters were washed two

times with 3 ml of ice-cold binding buffer, dried, and counted. Binding in the presence of 10 μ*M* piperoxan was considered nonspecific.

Autoradiography

Rat and hamster hearts were rapidly isolated after decapitation, and heart chambers were separated and snap frozen in prechilled isopentane. Cryostat whole heart sections (20 μm) from hamsters and four chambers of the rat heart were cut and mounted on acid-washed gelatinized slides, then placed overnight in a partial vacuum at −4°C. Slides were stored in boxes with Drierite (W.A. Hammond Drierite Co., Xenia, OH, U.S.A.) at −80°C until the autoradiographic procedures were performed.

Autoradiography was performed as previously described (16), using the same conditions determined for membrane binding. Duplicate slides were brought to room temperature in preincubation buffer containing 50 m*M* tromethamine hydrochloride, pH 7.7, and 0.1% polyethylenimine for 15 min to reduce nonspecific binding. The slides were then incubated with the ^{125}I-PIC prepared in binding buffer, for 1 h at room temperature. The incubation buffer consisted of 50 m*M* tromethamine hydrochloride, pH 7.7, 5 m*M* EDTA, 5 m*M* egtazic acid, 0.5 m*M* $MgCl_2$, and 50 μ*M* PMSF. Characterization of heart receptors was performed by binding of ^{125}I-PIC to adjacent sections under identical incubation conditions except for the irreversible inhibition of adrenergic receptors by incubating the sections for 35 min in 50 m*M* tromethamine hydrochloride (pH 7.7) in the presence of phenoxybenzamine (1 μ*M*) and ethylmaleimide (0.5 m*M*). Nonspecific binding was determined in the presence of 10 μ*M* of piperoxan.

Tissue sections were washed for 2 min each, with ice-cold incubation buffer, followed by two washes in 50 m*M* tromethamine hydrochloride buffer, pH 7.7, at 4°C, and finally dipped in distilled water to wash out salts. The slides were dried under a stream of cold air. The dried tissue sections were exposed in phosphor-sensitive cassette for 48 h, then scanned, visualized, and quantified by PhosphorImager (Image Quant, Molecular Dynamics, Sunnyvale, CA, U.S.A.).

Immunolocalization

Hearts were immediately excised, washed in fresh ice-cold phosphate-buffered saline (PBS), then fixed in 4% paraformaldehyde for 18 h at 4°C. After washing in PBS twice and overnight incubation in 0.5 *M* sucrose in PBS, the hearts were frozen in prechilled isopentane, then stored at −80°C until processed. Cryostat whole heart sections, 6–7 μm thick, were cut and mounted on poly-L-lysine-coated glass slides and placed overnight in a partial vacuum at 4°C.

Immunohistochemistry was performed as follows: slides were treated with 95% methanol/0.3% H_2O_2 for 30 min at 22°C, washed in PBS, and preincubated with 1% bovine serum albumin PBS for 30 min, followed by incubation with I receptor antibody or with nonimmune serum (donated by the late Dr. D. Reis, Weill Medical College of Cornell University, NY, U.S.A.) for the control sections. After washing with PBS, the sections were incubated with F(ab')2 fragments of horseradish peroxidase–conjugated goat anti-rabbit IgG at a dilution of 1:100 for 1 h at room temperature. The sections were reacted in 0.05% diaminobenzidine in 0.05 *M* tromethamine hydrochloride buffer (pH 7.4) in the presence of H_2O_2, then faintly counterstained with hematoxylin for light microscopy observation.

Immunoblotting

Immunoblotting was performed on cardiac tissues and compared with brain stem, used as a positive control, being the major site of imidazoline receptors. Denatured membrane protein samples (80 μg) were electrophoresed on 10% polyacrylamide gels followed by transfer to nitrocellulose (Hybond-P; Amersham, Arlington Heights, IL, U.S.A.). The membranes were incubated for 1 h with anti-imidazoline anti-serum diluted 1:1,000. Blots were washed, then incubated for 2 h with horseradish peroxidase–conjugated anti-rabbit IgG anti-serum diluted 1:5,000 in tromethamine-buffered saline and 5% milk. Immunoreactive bands were visualized by developing on film for 3 min as recommended by Amersham's enhanced chemiluminescence detection system (Amersham ECL Hyperfilm).

Drugs

^{125}I-PIC (2,200 Ci/mmol) was obtained from New England Nuclear (Boston, MA, U.S.A.) and stored at −20°C in ethanol. Moxonidine (donated by Solvay Pharmaceuticals, Hannover, Germany) was dissolved in 0.1 *M* of acetic acid. Efaroxan, rauwolscine, prazosin, IAA, phenoxybenzamine, ethylmaleimide, anandamide, and piperoxan (Sigma, St. Louis, MO, U.S.A.) were dissolved in water or ethanol as required, and (−)epinephrine was prepared in 0.001% ascorbic acid. All compounds were prepared daily and diluted in binding buffer to the required concentrations immediately before assay.

Data analysis

The equilibrium dissociation constant (Kd) and maximum binding capacity (Bmax) for the ligands used in the competitive binding radioreceptor studies were calcu-

lated by the iterative computerized nonlinear curve fitting method using the LIGAND computer program (Elsevier-Biosoft, Cambridge, U.K.). Data from human and hamster heart failure and hypertensive rats were compared with corresponding normal controls using the unpaired Student *t* test. Statistical significance was taken as $p < 0.05$. All data are reported as mean ± SEM.

RESULTS

The presence of imidazoline receptors in the heart was shown by immunohistochemistry, Western blot, membrane binding, and autoradiography. Immunolocalization of imidazoline receptors to normal rat heart was identified by the strong yellow staining observed in atria as compared with the nonimmune serum (Fig. 1) and faint staining in rat ventricles (not shown). Western blot analysis of rat brain stem and dog and human heart membranes using a specific anti-imidazoline receptor protein anti-serum showed the presence of at least five immunoreactive imidazoline receptor protein bands. The apparent molecular masses of these peptides were ~30, ~46, ~66, ~90, and ~150–200 (double-band) kD (Fig. 2). The strongest bands were observed in rat brain stem > human ventricles > dog ventricles > dog atria.

Membrane binding

The radiolabeled ligand ^{125}I-PIC binds with high affinity to both α_2-adrenergic and imidazoline I_1 receptors

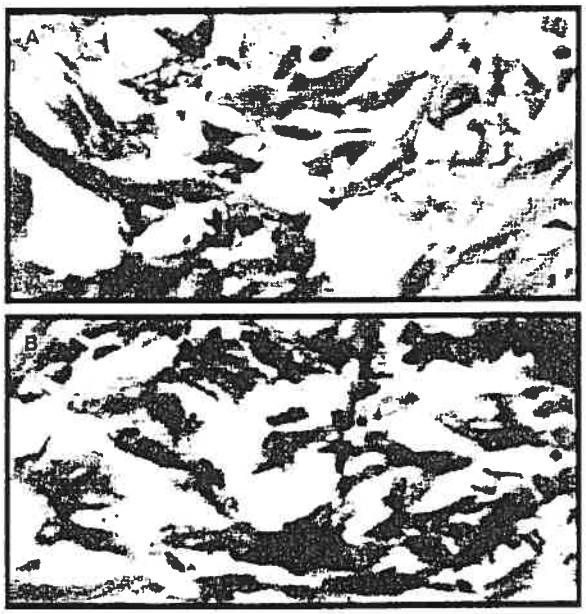

FIG. 1. Immunolocalization of imidazoline receptors in right atria of normotensive rats. A. Negative control. B. Imidazoline receptor antibody.

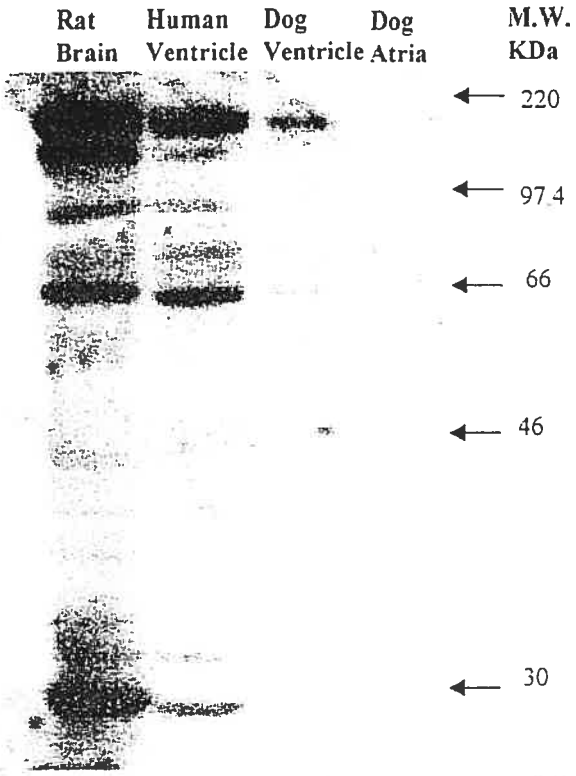

FIG. 2. Immunoblot analysis of imidazoline receptor protein in rat brain, dog atria and ventricles, and human ventricles.

in brain, platelets, and carotid bodies (17–20). Binding of ^{125}I-PIC to the heart membranes was rapid, specific, saturable, and of high affinity. Nonspecific binding determined in the presence of piperoxan was < 5%. Figure 3 illustrates curves plotted from data of binding assays of dog left atrial membranes represented as %B/Bo, where *B* and *Bo* represent, respectively, specific binding with and without competing drugs. In left atria, bound ^{125}I-PIC was progressively inhibited by increasing drug concentrations, so that at 10^{-5} *M* inhibition by moxonidine (imidazoline I_1 receptor agonist) represented 70%; epinephrine (α_2 agonists) 44%; rauwolscine (nonimidazoline α_2 antagonists) 47%; and only 20% by prazosin (α_1 antagonist). Similar inhibition profiles were obtained in ventricles, where binding was also inhibited by efaroxan (imidazoline receptor antagonist) and represented 50%. These results are consistent with ^{125}I-PIC binding to imidazoline I_1 and α_2-adrenergic, but not α_1-adrenergic receptors in the heart.

After irreversible blockade of α-adrenergic receptors, epinephrine and rauwolscine only minimally inhibited binding (range of 0–20%), confirming blockade of α_2-adrenergic receptors in the membrane preparations. Con-

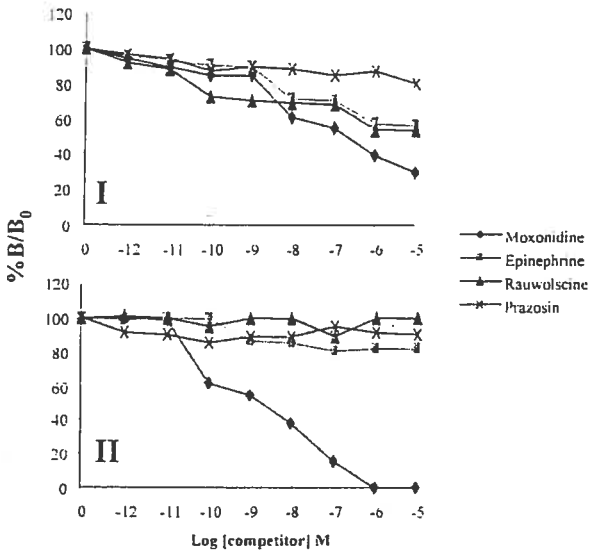

FIG. 3. Binding of ^{125}I-paraiodoclonidine (PIC) to dog left atrial membranes and inhibition of binding with α-adrenergic and imidazoline receptor agonists and antagonists. I = binding in the presence of α_2-adrenergic receptors; II = binding after irreversible blockade of α_2-adrenergic receptors with ethylmaleimide and phenoxybenzamine.

versely, moxonidine inhibited binding in a concentration-dependent manner, reaching total inhibition at 10^{-6} and 10^{-5} *M* concentrations (Fig. 3).

Heart imidazoline receptor kinetic parameters were calculated from curves obtained by moxonidine inhibition of binding in the absence of α_2-adrenergic receptors. Table 1 shows that dog left and right atrial membranes exhibited similar binding capacity, where Bmax represented 13.4 ± 1.7 and 20.1 ± 3.0 fmol/mg protein, with Kd = 3.9 ± 1.0 and 9.2 ± 1.4 n*M*, respectively. Imidazoline receptor binding was similar in left and right ventricular membranes (Bmax = 3.7 ± 0.6 and 5.0 ± 0.4 fmol/mg protein, respectively), but lower than those in atria. Receptor affinity was within the same nanomolar range (Kd = 2.6 ± 0.1 versus 4.0 ± 0.41 n*M*). Similarly, after α_2-adrenergic receptor blockade, imidazoline binding in sheep atrial membranes Bmax was 17.5 ± 3.5 fmol/mg protein, and Kd = 15.0 ± 1.1 n*M*; and in membranes from normal human ventricles, Bmax was 12.6 ± 1.3 fmol/mg protein, and Kd was 13.5 ± 0.6 n*M* (Table 1).

TABLE 1.
Kinetic parameters

	Bmax (fmol/mg protein)	Kd (nM)
Dog		
Left atria	13.4 ± 1.7	3.9 ± 1.0
Right atria	20.1 ± 3.0	9.2 ± 1.4
Left ventricles	3.7 ± 0.6	2.6 ± 0.1
Right ventricles	5.0 ± 0.4	4.0 ± 0.4
Sheep Atria	17.5 ± 3.5	15.0 ± 1.1
Human Ventricles	12.6 ± 1.3	13.5 ± 0.6

Kinetic parameters (Bmax, maximum binding capacity and Kd, dissociation constant) obtained by binding ^{125}I-PIC to atrial and ventricular membranes and inhibition of binding with increasing concentrations of moxonidine in the absence of α2-adrenergic receptors (see Methods).

Autoradiography

The results obtained from heart membrane binding studies were further confirmed by autoradiography performed on rat and hamster frozen heart sections. Binding of ^{125}I-PIC was equally inhibited (50%) by 10^{-6} *M* IAA (imidazoline I_1 receptor agonist) and 10^{-6} *M* epinephrine. Furthermore, ^{125}I-PIC binding to cannabinoid receptors, previously shown to be stimulated by I_1 receptor agonists (21), was verified using increasing concentrations of anandamide, a cannabinoid receptor agonist. In these sections, anandamide did not significantly affect binding, representing only 10 and 11% inhibition in left and right rat atria, respectively. These studies demonstrate that ^{125}I-PIC binding is mediated by imidazoline I_1 receptors and α_2-adrenergic receptors, but not by cannabinoid receptors. In further studies performed on hamster hearts after irreversible blockade of α_2-adrenergic receptors, ^{125}I-PIC binding was not inhibited by epinephrine but was inhibited by moxonidine in a concentration-dependent manner, reaching 70 ± 3% at concentrations of 10^{-5} *M*.

Receptor regulation

Figure 4 shows the effect of cardiovascular diseases on ^{125}I-PIC binding to rat and hamster hearts. Total specific ^{125}I-PIC binding to heart sections after α_2-adrenergic receptor blockade increased in SHR atria and cardiomyopathic (CMO) hamster hearts. Inhibition of binding by 10^{-5} *M* moxonidine represented 60 ± 4% of total binding in WKY atria and increased to 90 ± 2% in SHR atria. Compared with WKY, Bmax increased significantly ($n = 6$, $p < 0.001$) in SHR left (13.3 ± 0.6 versus 30.2 ± 4.1 fmol/unit area) and right (22.6 ± 0.3 versus 43.7 ± 4.4 fmol/unit area) atria, but no change occurred in ventricles. Kd was not altered by hypertension (Table 2).

In normal hamster hearts, imidazoline receptor Bmax was 13.9 ± 0.4 fmol/unit area and increased in cardiomyopathy to 26.0 ± 2.3 fmol/unit area ($p < 0.01$), whereas Kd of these tissues did not change (7.8 ± 0.4 versus 11.7 ± 1.4 n*M*). Similarly, imidazoline receptor total binding was increased by threefold in failing human

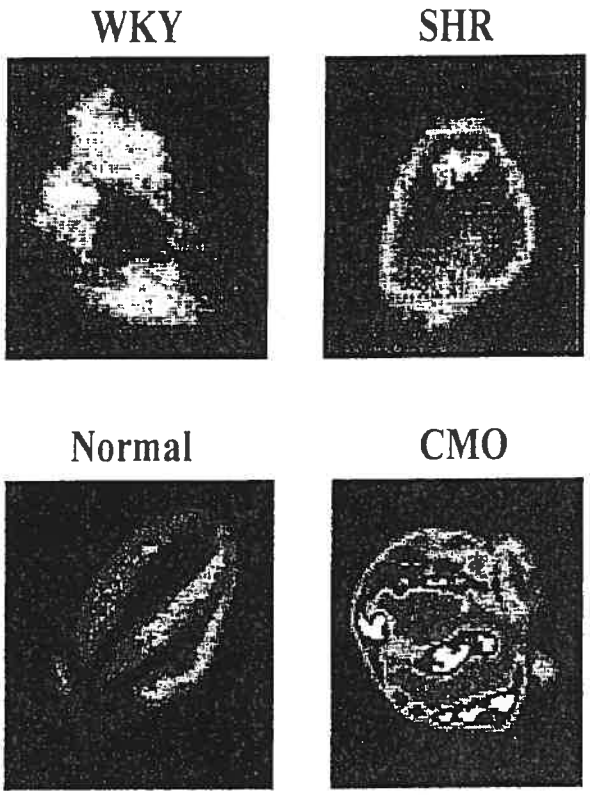

FIG. 4. Autoradiography of ^{125}I-paraiodoclonidine (PIC) binding in left atrial sections of spontaneously hypertensive (SHR) rats versus normotensive (Wistar-Kyoto) controls and in cardiomyopathic hamster (CMO) versus normal hamster hearts.

heart ventricular membranes. Figure 5 illustrates curves of ^{125}I-PIC binding to human ventricular membranes after blockade of α_2-adrenergic receptors and inhibition of binding by competing drugs. Inhibition of binding by moxonidine and IAA represented 70 ± 4% and 51 ± 2%, respectively, in the normal human ventricles, and increased to 95 ± 1% and 85 ± 2% in the failing heart membranes, indicating receptor upregulation. This upregulation was due to an increased Bmax (12.6 ± 1.3 versus 35.5 ± 2.9 fmol/mg protein, n = 3, $p < 0.003$), but not affinity.

TABLE 2.
Regulation of imidazoline receptor kinetics

	Bmax (fmol/mg protein)	Kd (nM)
Rat, left atria		
WKY	13.3 ± 0.6	4.8 ± 0.4
SHR	30.2 ± 4.1*	3.9 ± 0.7
Rat, right atria		
WKY	22.6 ± 0.3	4.5 ± 0.3
SHR	43.7 ± 4.4*	7.2 ± 0.8
Hamster heart		
Normal	13.9 ± 0.4	7.8 ± 0.4
CMO	26.0 ± 2.3	11.7 ± 1.4

Regulation of imidazoline receptor kinetics in rat hypertension and human and hamster heart failure (see Methods). *$p < 0.01$ vs corresponding control.

DISCUSSION

This study consists of two main parts: identification of imidazoline receptors in the heart and receptor regulation in cardiovascular diseases. Qualitative demonstration of imidazoline receptors in the heart was achieved by immunolocalization using the polyclonal anti-serum to an I receptor binding protein isolated from bovine adrenal chromaffin cells. This protein specifically labels imidazoline receptor protein in rat brain, rat aortic vascular smooth muscle cells, bovine pulmonary artery endothelial cells, and human brain. The antibody recognizes the ~70-, ~45-, and ~29-kD imidazoline receptor subtypes but not α_2-adrenergic receptors (22).

Using the same antibody, Western blot analysis of atrial and ventricular heart membranes showed multiple molecular mass peptides, similar to those thus far described in the brain but in much lower quantities. This is consistent with the finding that a partial cDNA clone detected by imidazoline receptor-selective anti-sera is present in the human heart, albeit in much lower levels than those in pituitary and brain (23). However, we presently have no explanation as to the appearance of bands corresponding to high-molecular-mass peptides in samples from brain stem, ventricles, and atria, except possible dimerization of the 85-kD peptide, reported as the full-length imidazoline receptor protein (24).

Because the receptor antibody does not discriminate between I_1 and I_2 receptors, competitive membrane binding and autoradiography techniques were used using the radiolabeled ligand (^{125}I-PIC), which binds with high affinity to α_2-adrenergic receptors and imidazoline I_1 receptors but with low affinity to I_2 receptors (19). In these studies, identification of imidazoline I_1 receptors was achieved using optimal conditions that favor imidazoline over α_2-adrenergic receptor binding, such as low $MgCl_2$ buffer (25). Furthermore, binding was performed with and without blockade of adrenergic receptors, by using a combination of ethylmaleimide, which inhibits specific binding of ^{125}I-PIC to α_2-adrenergic receptors, and phenoxybenzamine, which irreversibly blocks α_2- and α_1-adrenergic receptors at high potency but has minimal effects on imidazoline receptors (11,26). Specific binding to I_1 receptors was determined using moxonidine,

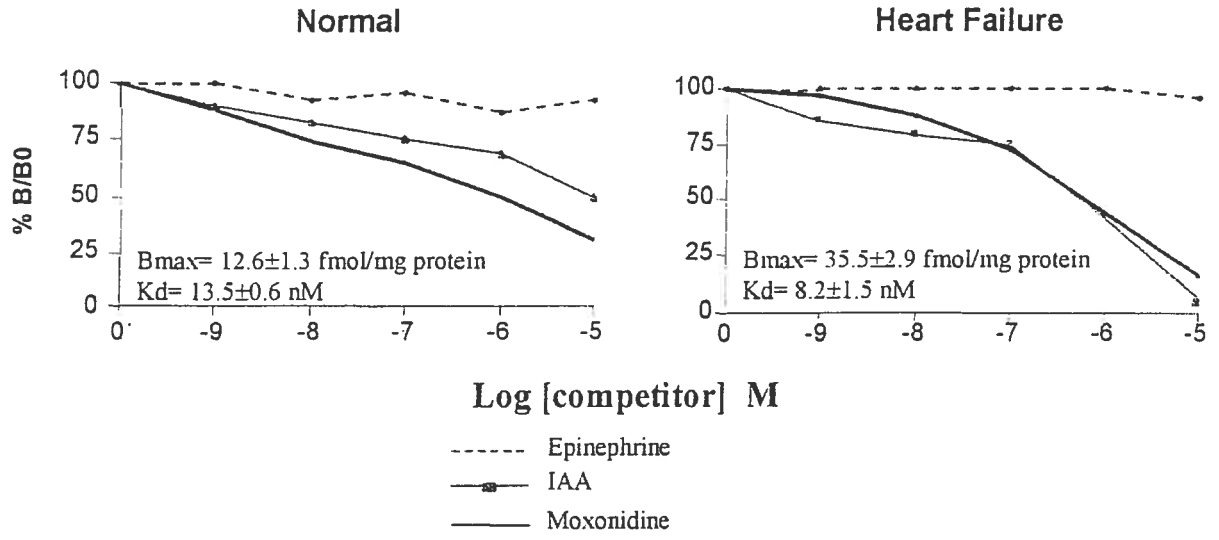

FIG. 5. Binding of ^{125}I-paraiodoclonidine (PIC) to human ventricular membranes obtained from normal and heart failure patients, after irreversible blockade of α_2-adrenergic receptors with ethylmaleimide and phenoxybenzamine and inhibition of binding with moxonidine, imidazole-4-acetic acid (IAA), and epinephrine.

which shows a 100- and 700-fold selectivity for the I_1 imidazoline receptor over α_{2b}-adrenergic receptors in rat brain and renal medullary membranes, respectively (9,19).

^{125}I-PIC binding was observed in dog heart membranes and hamster and rat heart sections. Binding was inhibited by IAA and epinephrine, implying that the heart exhibits both imidazoline I_1- and α_2-adrenergic receptors, respectively. After irreversible inhibition of adrenergic receptors, binding of ^{125}I-PIC was no longer inhibited by increasing concentrations of prazosin, epinephrine, and rawoulscine, confirming total inhibition of α-adrenergic receptors. Conversely, concentration-dependent inhibition of binding with moxonidine, efaroxan, and IAA indicated the presence of specific I_1 receptors, and the lack of inhibition with anandamide confirmed that ^{125}I-PIC did not bind to cannabinoid receptors that have been proposed to bind moxonidine (21).

Calculation of kinetic parameters in heart membranes from sheep, dog, and human, or rat and hamster hearts revealed that the affinity of I_1 receptors in the heart is in the nanomolar range, similar to those reported for other tissues using ^{3}H-clonidine, such as bovine, rat, and human brain stem (6–7 n*M*) (27) and bovine adrenomedullary cells (Kd 12 n*M*) (28). The imidazoline receptor binding capacity is twofold greater in atria than ventricles but at least-10 fold lower than that reported by Ernsberger et al. (19) in ventrolateral medulla (VLM) membranes, the primary site of imidazoline binding.

The second aim of the study addressed the regulation of heart I_1 receptors. Again, membrane binding or autoradiography revealed a twofold increase of imidazoline sites in atria of SHRs, a threefold increase in failing human ventricles, and a twofold increase in hearts of hamsters with advanced cardiomyopathy. These data indicate that imidazoline I_1 receptors in cardiac tissue are altered in cardiovascular diseases; and therefore, it is reasonable to suggest that heart imidazoline I_1 receptors are involved in cardiovascular regulation.

The mechanisms of heart I receptor regulation in cardiovascular diseases are beyond the scope of this study. However, several studies have shown that imidazoline sites are subject to physiologic and pharmacologic regulation and that I receptor regulation is tissue specific. Imidazoline receptor upregulation has been reported in postmortem brains of suicide victims (29), in platelets of patients with depression (30), and platelets of postmenopausal women (31). Chronic treatment with the anti-depressant drug imipramine downregulates I_1 imidazoline receptors in rat brain stem (32). Chronic administration of idazoxan to rabbits causes a substantial decrease in the number of renal imidazoline binding sites (33) but increases the density of central I receptors (34). I receptor binding is also upregulated in kidneys of genetically hypertensive rats (35).

Regardless of the mechanisms of regulation, upregulated heart imidazoline receptors in cardiovascular diseases characterized by sympathetic efferent neuronal overactivity may reflect the benefits of imidazoline receptor agonists observed in patients as compared with normal subjects. Heart imidazoline receptors may play

an important role in normal cardiovascular regulation, especially because agmatine, an endogenous ligand for imidazoline receptors, is present in the heart (36), and because clonidine (imidazoline I_1 and α_2-adrenergic receptor agonist) stimulates the release of ANP from isolated rat hearts (37). ANP is a cardiac hormone that plays an integral role in volume and pressure homeostasis in normal and pathophysiologic conditions. Circulating ANP levels are increased in hypertension and heart failure; interestingly, in parallel to the present finding of upregulated imidazoline receptors in the heart.

In conclusion, this study demonstrates the presence of imidazoline I_1 receptors in the heart and shows that these receptors are upregulated in cardiovascular diseases. Heart imidazoline receptors may be important in the regulation of cardiovascular function, acting locally to exert cardioprotective effects, similarly to their reported neuroprotective (22) and nephroprotective effects (38).

Acknowledgments: This work was supported by grants (to.SMD and JG) from the Canadian Institutes for Health Research (MO-15417) and the Heart and Stroke Foundation of Canada.

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Normalization of Up-Regulated Cardiac Imidazoline I_1-Receptors and Natriuretic Peptides by Chronic Treatment with Moxonidine in Spontaneously Hypertensive Rats

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ABSTRACT: The effect of treatment with moxonidine (120 μg/kg/h sc, 4 weeks) on cardiac I_1-receptors and natriuretic peptide synthesis was evaluated in spontaneously hypertensive rats (SHR). I_1-receptor protein (85 kD) was up-regulated in SHR atria, and normalized in right and left atria by moxonidine. Similarly, moxonidine normalized atrial and ventricular atrial natriuretic peptide messenger RNA (mRNA) and brain natriuretic peptide mRNA. This study shows that cardiac I_1-receptors are functional, being regulated by hypertension and by chronic exposure to agonist, and that cardiac natriuretic peptides may be regulated by I_1-receptor-mediated mechanisms.

KEYWORDS: imidazoline receptors; moxonidine; natriuretic peptides

INTRODUCTION

Imidazoline I_1-receptors are non-adrenergic and non-cholinergic neurotransmitter receptors present in the brainstem, adrenal chromaffin cells, and kidneys. Activation of I_1-receptors is associated with blood pressure reduction, primarily by sympathoinhibition[1,2]. In addition, we have recently identified imidazoline I_1-receptors in the heart atria and ventricles, and shown that heart imidazoline receptors are up-regulated in cardiovascular diseases, such as hypertension and heart failure,[3] implying that heart imidazoline receptors may be functional and involved in cardiovascular regulation.

Moxonidine, an antihypertensive imidazoline compound, reduces blood pressure by activation of central and peripheral imidazoline I_1-receptors and subsequent decrease of sympathetic nervous activity and stimulation of renal actions.[2] Acute moxonidine injections in normotensive Sprague-Dawley (SD) rats dose-dependently increase diuresis, natriuresis, and cyclic guanosine 3′,5′-monophosphate (cGMP) excretion as well as plasma levels of natriuretic peptides,[4] cardiac hormones that reduce blood pressure by several mechanisms, including vasodilation, diuresis, natriuresis, and sympathoinhibition.

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Based on the findings that cardiac natriuretic peptides may be involved in the acute activation of imidazoline receptors by moxonidine,[4] and that both imidazoline receptors and natriuretic peptide synthesis are increased in hearts of hypertensive rats,[3,5] the following studies were performed. We investigated the effect of a one-month treatment with moxonidine on imidazoline receptors and natriuretic peptide expression in hypertensive rat hearts, with the aim of demonstrating functionality of cardiac imidazoline I_1-receptors.

METHODS

Female spontaneously hypertensive rats (SHR; 12–14 weeks old) were treated during one month with two doses of moxonidine (60, 120 μg/kg/h) or saline vehicle, via Alzet osmotic minipumps (2ML4) implanted under the neck skin. Sprague-Dawley rats (200–225 g) served as normotensive controls. Animals were housed in a temperature- and light-controlled room with food and water *ad libitum*. After one month of treatment, the rats were sacrificed by decapitation and the hearts were rapidly isolated, divided into four compartments, then flash frozen in liquid N_2 and stored at –80°C.

Imidazoline receptor regulation in cardiac atria and ventricles was analyzed by Western blot using a polyclonal imidazoline receptor antibody, as we have previously described.[3] Total RNA from ventricles and atria were extracted, then reverse-transcribed into cDNA and subjected to semiquantitative polymerase chain reaction (PCR), using specific primers for atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH).[5] Values normalized to corresponding GAPDH are reported as percent of normotensive controls or vehicle-treated SHR controls.

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RESULTS

Immunoblotting revealed that, compared to normotensive controls (100%), the intensity of the bands that correspond to 85 kD was increased (135 ± 3%; $n = 10$; $P < 0.001$) in SHR atria. Compared to vehicle-treated SHR (100%), treatment with 60 and 120 μg moxonidine significantly ($n = 6$–10; $P < 0.001$) decreased the intensity of the 85 kD band in right atria to 51 ± 2% and 47 ± 3%, respectively. The bands that correspond to 29/30 kD were not altered in SHR with or without treatment (FIG. 1). The intensity of the bands corresponding to 29/30 and 85 kD proteins were not increased in hypertensive rat ventricles. One-month treatment with two doses of moxonidine had no effect on the 85 kD band, but was associated with reduced intensity of the bands corresponding to 29/30 kD to 89 ± 2% of vehicle-treated SHR ($n = 6$; $P < 0.002$).

ANP mRNA decreased in right atria and left ventricles to 68 ± 2% and 70 ± 6% of corresponding vehicle-treated hypertensive controls, respectively ($P < 0.001$). Similarly, right atrial and left ventricular BNP mRNA decreased to 46 ± 1% and 65 ± 1% ($P < 0.001$; FIG. 2). The levels of ANP and BNP mRNA were not significantly different from those measured in normotensive controls.

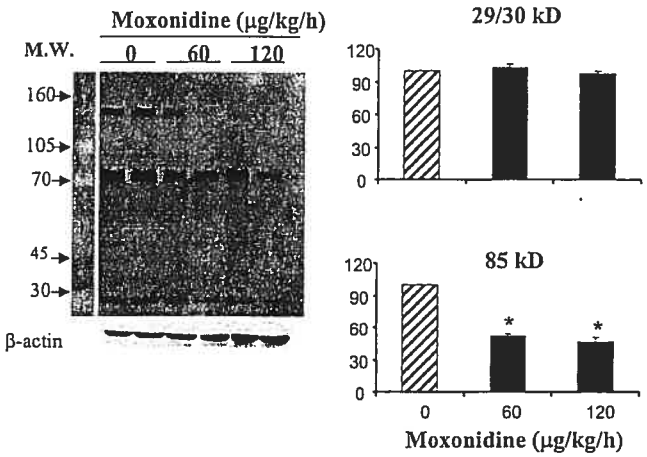

FIGURE 1. Western blot analysis of imidazoline receptor proteins in right atria in response to one-month treatment with two doses of moxonidine.

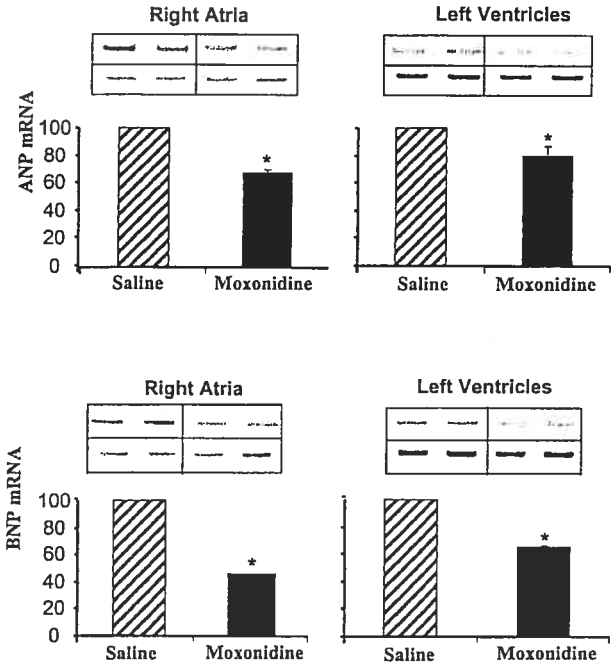

FIGURE 2. Effect of moxonidine treatment on natriuretic peptides ANP and BNP mRNA in right atria of SHR. Results are normalized to corresponding GAPDH and reported as percent of vehicle-treated SHR.

DISCUSSION

This study shows that chronic one-month treatment of hypertensive rats with moxonidine is associated with normalization of up-regulated imidazoline receptors and natriuretic peptide synthesis in cardiac atria and ventricles.

Imidazoline receptors of 29/30 kD, 45 kD, 85 kD, 176 kD proteins have been isolated from brainstem RVLM.[6] Similar proteins are also present in the heart.[3] However, it is not yet known which protein corresponds to the functional imidazoline I_1-receptor. Based on our present finding, we suggest that functional cardiac imidazoline I_1-receptors are tissue-specific, being differentially regulated in atria and ventricles by hypertension and chronic exposure to agonist. Whereas the 85 kD protein may correspond to the functional I_1-receptor in atria, it may be the 29/30 kD protein that is functional in ventricles. However, further studies are required to confirm this observation.

Previous studies from our laboratory and others have shown that natriuretic peptide synthesis is increased in hypertensive rat hearts.[3,7,8] In the present study, chronic treatment of SHR with 60 and 120 μg/kg/h moxonidine, over one month, is associated with significantly reduced ANP mRNA and BNP mRNA in right atria and left ventricles as compared to corresponding vehicle-treated SHR. The mechanisms of natriuretic peptide decrease by chronic moxonidine have not been investigated, but these changes parallel the changes in imidazoline receptor proteins; both are up-regulated by hypertension and down-regulated by moxonidine treatment. Therefore, based on the present findings and on our previous results that acute moxonidine injections increase circulating ANP levels, it appears likely that cardiac natriuretic peptides may be regulated by imidazoline I_1-receptor-mediated mechanisms.

Q: Please spell out RVLM.

ACKNOWLEDGMENTS

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Q: City of manufacturer?

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Imidazoline Receptors but Not α_2-Adrenoceptors Are Regulated in Spontaneously Hypertensive Rat Heart by Chronic Moxonidine Treatment

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ABSTRACT

We have recently identified imidazoline I_1-receptors in the heart. In the present study, we tested regulation of cardiac I_1-receptors versus α_2-adrenoceptors in response to hypertension and to chronic exposure to agonist. Spontaneously hypertensive rats (SHR, 12–14 weeks old) received moxonidine (10, 60, and 120 μg/kg/h s.c.) for 1 and 4 weeks. Autoradiographic binding of ^{125}I-paraiodoclonidine (0.5 nM, 1 h, 22°C) and inhibition of binding with epinephrine (10^{-10}–10^{-5} M) demonstrated the presence of α_2-adrenoceptors in heart atria and ventricles. Immunoblotting and reverse transcription-polymerase chain reaction identified α_{2A}-, α_{2B}-, and α_{2C}-adrenoceptor proteins and mRNA, respectively. However, compared with normotensive controls, cardiac α_2-adrenoceptor kinetic parameters, receptor proteins, and mRNAs were not altered in SHR with or without moxonidine treatment. In contrast, autoradiography showed that up-regulated atrial I_1-receptors in SHR are dose-dependently normalized by 1 week, with no additional effect after 4 weeks of treatment. Moxonidine (120 μg/kg/h) decreased B_{max} in right (40.0 ± 2.9–7.0 ± 0.6 fmol/unit area; $p < 0.01$) and left (27.7 ± 2.8–7.1 ± 0.4 fmol/unit area; $p < 0.01$) atria, and decreased the 85- and 29-kDa imidazoline receptor protein bands, in right atria, to 51.8 ± 3.0% ($p < 0.01$) and 82.7 ± 5.2% ($p < 0.03$) of vehicle-treated SHR, respectively. Moxonidine-associated percentage of decrease in B_{max} only correlated with the 85-kDa protein ($R^2 = 0.57$; $p < 0.006$), suggesting that this protein may represent I_1-receptors. The weak but significant correlation between the two imidazoline receptor proteins ($R^2 = 0.28$; $p < 0.03$) implies that they arise from the same gene. In conclusion, the heart possesses I_1-receptors and α_2-adrenoceptors, but only I_1-receptors are responsive to hypertension and to chronic in vivo treatment with a selective I_1-receptor agonist.

Most of the centrally acting antihypertensive drugs, such as clonidine and related imidazoline derivatives, mediate sympathoinhibition, not only via activation of central nervous α_2-adrenoceptors but also via imidazoline I_1-receptors (Bricca et al., 1989; Bousquet, 1997). Imidazoline I_1-receptors are nonadrenergic and noncholinergic neurotransmitter receptors that possess low affinity for norepinephrine and other catecholamines. I_1-receptors are mainly found in the brainstem, adrenal chromaffin cells, and kidneys. In addition, we have recently identified I_1-receptors in heart atria and ventricles and shown that atrial I_1-receptors are up-regulated in rat hypertension and ventricular I_1-receptors are up-regulated in human and hamster heart failure (El-Ayoubi et al., 2002a). In other studies, we demonstrated that acute injections of moxonidine, an imidazoline compound that shows 40 times higher affinity to I_1-receptor versus α_2-adrenoceptors, are associated with enhanced release of atrial natriuretic peptide (Mukaddam-Daher and Gutkowska, 2000), a cardiac hormone involved in pressure and volume homeostasis. Together, these studies led us to suggest that heart I_1-receptors are functional and may be involved in cardiovascular regulation.

Previous binding studies reported [^{3}H]idazoxan binding sites (I_2-receptors) but not I_1-receptors in human atrial appendage; but functionally, these receptors were different from presynaptic imidazoline receptors implicated in inhibition of noradrenaline release. Accordingly, atrial presynaptic imidazoline receptors were considered non-I_1 non-I_2 receptors, and the effects of moxonidine to inhibit noradrenaline

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ABBREVIATIONS: SHR, spontaneously hypertensive rats; WKY, Wistar-Kyoto; SD, Sprague-Dawley; RT-PCR, reverse transcription-polymerase chain reaction; PIC, paraiodoclonidine; PCR, polymerase chain reaction.

release in atrial appendages were attributed to presynaptic α_2-adrenoceptors (Molderings and Gothert, 1999). In contrast, consistent with the presence of I_1-receptors in the heart, Schäfer et al. (2002) have recently shown in isolated perfused rats hearts that moxonidine is able to decrease noradrenaline release independently of α_2-adrenoceptors.

In fact, functional separation between imidazoline I_1-receptors and α_2-adrenoceptors is rather difficult, because these receptors are often colocalized and ligands with affinity to imidazoline I_1-receptors also bind to α_2-adrenoceptors (Bousquet, 1997). However, previous studies indicate that imidazoline receptors and α_2-adrenoceptors are subject to pathophysiological and pharmacological regulation (Yakubu et al., 1990; Ernsberger et al., 1991; Zhu et al., 1997; Ivanov et al., 1998). Therefore, the aim of the present studies was to test regulation of cardiac I_1-receptors versus α_2-adrenoceptors, by showing that I_1-receptors, but not α_2-adrenoceptors are regulated in hypertension and in response to exposure to agonist. Accordingly, studies were performed to 1) demonstrate the presence of α_2-adrenoceptors in the heart and their possible regulation in hypertension, and 2) investigate the effect of chronic in vivo exposure to moxonidine on I_1-receptors and α_2-adrenoceptors in hearts of normotensive rats and spontaneously hypertensive rats (SHR) with established hypertension.

Materials and Methods

Female SHR (12–14 weeks old) with established hypertension and age-matched normotensive Wistar-Kyoto (WKY) and Sprague-Dawley (SD) rats were purchased from Charles River (St. Constant, QC, Canada). Animals were housed in a temperature- and light-controlled room with food and water ad libitum, and maintained for at least 3 days before experimentation. Experiments were performed following the approval of the Bioethics Committee of Centre Hospitalier de L'Université de Montréal, according to the Canadian Guidelines.

Alzet osmotic mini-pumps (2ML1 and 2ML4; Alzet, Cupertino, CA) were implanted subcutaneously in SHR, under isoflurane anesthesia, as we have described previously (Menaouar et al., 2002). These mini-pumps allowed continuous delivery of moxonidine (generous gift from Solvay Pharmaceuticals, Hannover, Germany) or saline vehicle at the rate of 10 μl/h (2ML1), for 1 week, and 2.5 μl/h (2ML4) for 4 weeks. The concentrations of moxonidine were adjusted to allow delivery of 10, 60, and 120 μg/kg/h. The solution of moxonidine was prepared by dissolving the drug in isotonic saline, pH <6.5, and then pH adjusted to 7.0 to 7.4 by NaOH. Rats were sacrificed after 1 and 4 weeks of vehicle and moxonidine treatment, and heart atria and ventricles were separated, snap-frozen in prechilled isopentane, and then stored at −80°C, for receptor analysis by autoradiographic binding, immunoblotting, and RT-PCR.

To rule out the influence of blood pressure on receptor regulation, another group of SHR was treated with hydralazine, given at 30 mg/kg/day, in drinking water, for 1 week. The effectiveness of hydralazine was verified by tail cuff measurement of systolic blood pressure before and after 1-week treatment. Then, rats were sacrificed and tissues collected as described above.

Autoradiography. Autoradiography of heart I_1-receptors and α_2-adrenoceptors was performed on frozen heart sections from WKY and SD rats and from saline- and moxonidine-treated SHR, using radiolabeled paraiodoclonidine (^{125}I-PIC; 2200 Ci/mmol; PerkinElmer Life and Analytical Sciences, Boston, MA) as we have described previously (El-Ayoubi et al., 2002a). Because ^{125}I-PIC binds to both receptor types, autoradiography was performed, separately, in conditions that favor α_2-adrenoceptor binding and in conditions that favor I_1-receptor binding. For α_2-adrenoceptors, the slides were incubated for 1 h at 22°C with 0.5 nM ^{125}I-PIC in incubation buffer: 50 mM Tris-HCl (pH 7.7), 5 mM EDTA, 5 mM EGTA, 10 mM $MgCl_2$, and 50 μM phenylmethylsulfonyl fluoride. Binding was inhibited by increasing concentrations of epinephrine (10^{-10}–10^{-5} M). Binding in the presence of 10^{-4} M piperoxan was considered nonspecific. After several washes, the slides were dried, exposed in phosphor-sensitive cassette for 48 h, and then scanned, visualized, and quantified by PhosphorImager (ImageQuant; Amersham Biosciences Inc., Piscataway, NJ).

Autoradiography for I_1-receptors was performed under identical incubation conditions, except for prior incubation of slides with 1 mM phenoxybenzamine and 0.5 mM ethylmaleimide for 35 min at room temperature, to irreversibly inhibit adrenoceptor binding; and by decreasing the concentration of $MgCl_2$ in the incubation buffer to 0.5 mM, conditions that favor binding to I_1-receptors (Ernsberger et al., 1995). Binding of ^{125}I-PIC was competitively inhibited by increasing concentrations (10^{-12}–10^{-5} M) of moxonidine

Membrane Preparation and Immunoblotting. Membranes of ventricular and atrial tissues were prepared in sucrose buffer as described previously (El-Ayoubi et al., 2002a). Protein content was measured spectrophotometrically, using bovine serum albumin as standard.

Immunoblotting was performed (El-Ayoubi et al., 2002a) using 30 μg of denatured protein samples from cardiac tissues and incubation of blots with rat α_{2A}-, α_{2B}-, and α_{2C}-adrenoceptor antiserum (1:500; Santa Cruz Biotechnology, Inc., Santa Cruz, CA), or anti-imidazoline receptor antiserum and nonimmune antiserum (generous gift from S. Regunathan, Department of Psychiatry, University of Mississippi Medical Center, Jackson, MS) diluted 1:1000, or with anti-β-actin (1:500). The blots were incubated with horseradish peroxidase-conjugated anti-rabbit IgG antiserum (1:5000). Immunoreactive bands were visualized by enhanced chemiluminescence detection system (ECL hyperfilm; Amersham Biosciences Inc.), according to the manufacturer's instructions.

Total RNA Extraction and RT-PCR. Total RNA was extracted from the rat heart tissues using TRIzol reagent (Invitrogen, Carlsbad, CA) according to the protocol described by the manufacturer. PCR reactions were performed (Zou and Cowley, 2000) using specific primer pairs for rat α_{2A}-, α_{2B}-, and α_{2C}-receptors, or β-actin (QIAGEN Operon, Alameda, CA). After electrophoresis on agarose gel in the presence of ethidium bromide, fluorescent PCR products were scanned, counted, and analyzed with the ImageQuant software. These data were normalized to the corresponding values of β-actin PCR product in the same samples.

Data Analysis. The equilibrium dissociation constant (K_d) and maximum binding capacity (B_{max}) for the ligands used in autoradiography were calculated by the nonlinear method using the Ligand computer program (Elsevier-Biosoft, Cambridge, UK). Densitometric measurements of immunoblots were performed using Scion computer program (National Institutes of Health, Bethesda, MD). Correlation coefficients were calculated from linear regression (GraphPad Prism; GraphPad Software, Inc., San Diego, CA). Differences in data obtained from vehicle- or moxonidine-treated rats were compared by nonpaired Student's t test. $P < 0.05$ was considered significant. All data are expressed as mean ± S.E.M.

Results

Cardiac α_2-Adrenoceptors. Autoradiographic binding of ^{125}I-PIC to heart atrial and ventricular sections was inhibited by increasing concentrations of epinephrine. Kinetic parameters obtained from competitive inhibition curves (Table 1) revealed that α_2-adrenoceptor affinity ($K_d \cong 2.5$ nM) and B_{max} in right atria (12.8 ± 0.7 versus 13.4 ± 0.9 fmol/unit area), left atria (12.8 ± 0.4 versus 11.7 ± 0.7 fmol/unit area) and left ventricles (11.7 ± 1.1 versus 12.2 ± 0.5 fmol/unit area) were not altered in SHR compared with WKY rats.

TABLE 1
Kinetic parameters of cardiac α_2-adrenoceptors

	WKY	SHR		
Moxonidine (μg/kg/h)	0	0	60	120
Right atria				
B_{max} (fmol/unit area)	12.8 ± 0.7	13.4 ± 0.9	11.5 ± 0.7	11.9 ± 0.9
K_d (nM)	2.3 ± 0.1	2.4 ± 0.3	2.2 ± 0.3	2.3 ± 0.3
Left atria				
B_{max} (fmol/unit area)	12.8 ± 0.4	11.7 ± 0.7	11.9 ± 0.7	12.5 ± 0.3
K_d (nM)	2.5 ± 0.3	2.1 ± 0.1	3.3 ± 0.5	2.2 ± 0.2
Left ventricles				
B_{max} (fmol/unit area)	11.7 ± 1.1	12.2 ± 0.5	11.6 ± 0.6	12.0 ± 0.4
K_d (nM)	2.5 ± 0.5	2.3 ± 0.3	2.9 ± 0.2	2.3 ± 0.4

Binding to cardiac α_2-adrenoceptors in SHR was also not altered by chronic in vivo moxonidine treatment. B_{max} remained in 120 μg/kg/h moxonidine-treated SHR right atria at 11.9 ± 0.9 fmol/unit area and K_d at 2.3 ± 0.3 nM. Similarly, kinetic parameters obtained in left atria and left ventricles were not altered in vehicle- or moxonidine-treated SHR (Table 1).

Three α_2-adrenoceptor subtypes were identified in cardiac tissues of SD and SHR, by immunoblotting. Densitometric measurements of the bands corresponding to α_{2A}- (Fig. 1), α_{2B}-, and α_{2C}-adrenoceptors (data not shown) were not significantly different in right atria and left ventricles of SHR versus SD, nor in vehicle- and moxonidine-treated SHR, where variation did not exceed 10%. Furthermore, levels of three subtypes of α_2-adrenoceptor mRNA detected in right and left atria and left ventricles of SHR were also not significantly different among vehicle- or moxonidine-treated groups, where variation did not exceed 10% (Fig. 2).

Cardiac Imidazoline Receptors. Autoradiography showed that total specific binding of ^{125}I-PIC to I_1-receptors was higher in SHR atria (162%) compared with normotensive WKY rats, considered as 100%. Also, total specific binding in atria decreased after treatment with moxonidine at 10, 60, and 120 μg/kg/h for 1 week (Fig. 3).

Competitive inhibition curves were plotted from values obtained from normotensive and hypertensive vehicle- and moxonidine-treated rats and presented as percentage of B/B_0, where B and B_0 represent, respectively, binding with and without moxonidine (Fig. 3). Kinetic parameters calculated from these curves using the Ligand computer program revealed that 1-week treatment dose dependently decreased B_{max} in SHR right and left atria. At the lowest dose of 10 μg of moxonidine, B_{max} decreased from 40.0 ± 2.9 to 18.2 ± 0.4 fmol/unit area ($p < 0.01$) in right atria and from 27.7 ± 2.8 to 12.3 ± 0.6 fmol/unit area ($p < 0.04$) in left atria. The doses of 60 and 120 μg moxonidine decreased B_{max} in rat right and left atria to values not significantly different from two normotensive controls (Table 2). Four-week treatment did not have additional effects, so that at 120 μg of moxonidine, B_{max} in right atria represented 9.0 ± 0.3 fmol/unit area. Moxonidine treatment did not affect B_{max} and K_d of I_1-receptors in right and left ventricles of moxonidine- and vehicle-treated SHR (Table 2).

The presence of three immunoreactive imidazoline receptor protein bands was shown in cardiac tissues by immunoblotting. The apparent molecular masses of these proteins were around 160, 85, and 29/30 kDa. Densitometric measurements of bands corresponding to the 160-kDa band was only slightly increased in atria of SHR, and almost not detected in normotensive SD rats and in moxonidine-treated SHR for 1 or 4 weeks. On the other hand, the density of bands corresponding to 85-kDa protein increased significantly ($p < 0.05$)

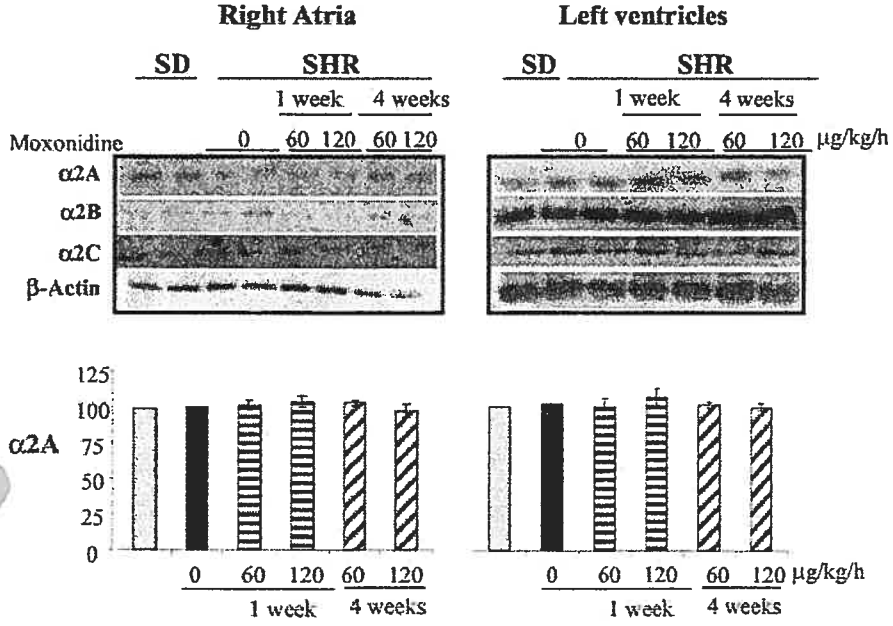

Fig. 1. Representative immunoblot of α_2-adrenoceptor subtypes (α_{2A}, α_{2B}, and α_{2C}) and β-actin, and densitometric data of α_{2A} measured in right atria and left ventricles of SD and SHR after treatment with moxonidine (0, 60, and 120 μg/kg/h) for 1 and 4 weeks. Data normalized to corresponding β-actin are presented as percentage of change from SD and vehicle-treated SHR.

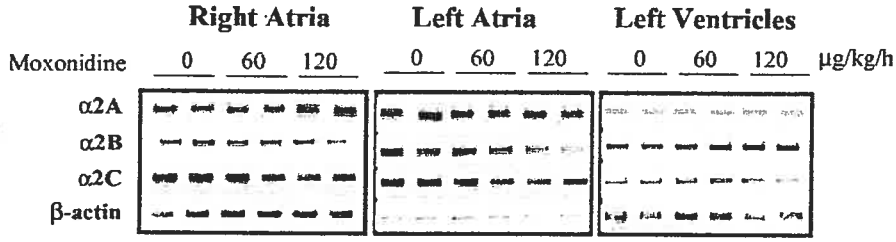

Fig. 2. RT-PCR mRNA products of α_2-adrenoceptors and β-actin in right and left atria and left ventricles of SHR treated with moxonidine (0, 60, and 120 μg/kg/h) for 4 weeks.

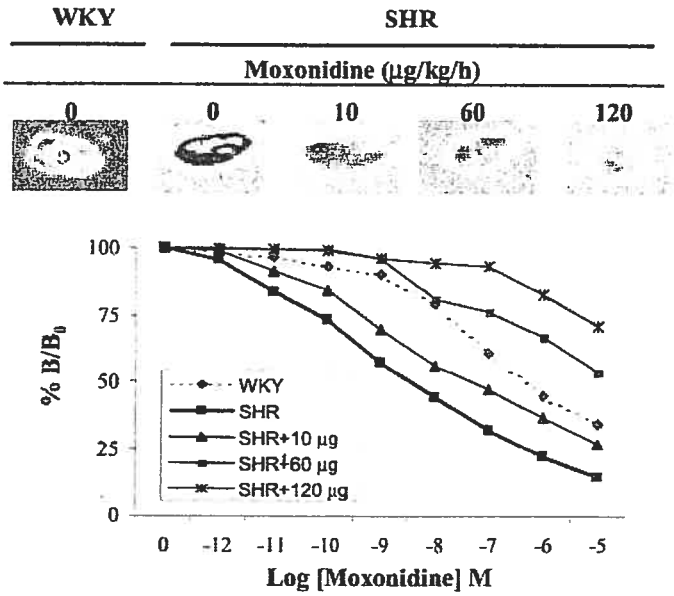

Fig. 3. Top, representative autoradiography of total ^{125}I-PIC binding to right atrial tissue sections (after irreversible inhibition of α-adrenoceptor binding) in WKY and SHR after 1-week treatment with moxonidine (0, 10, 60, and 120 μg/kg/h). Bottom, specific binding of ^{125}I-PIC to right atrial sections in WKY and SHR after 1-week treatment with moxonidine (0, 10, 60, 120 μg/kg/h). Data are presented as $\%B/B_0$, where B and B_0 represent binding in the absence and presence of increasing concentrations (10^{-12}–10^{-5} M) of inhibiting ligand.

in right atria of SHR to 134.6 ± 3.3% compared with normotensive control (considered as 100%).

Figure 4 shows that, compared with vehicle-treated SHR (considered as 100%), chronic moxonidine treatment resulted in a significant ($p < 0.01$) decrease in the intensity of the bands corresponding to 85-kDa proteins to represent 83.4 ± 1.9, 59.9 ± 2.7, and 51.8 ± 3.0% in 10, 60, and 120 μg moxonidine-treated SHR for 1 week, respectively. Treatment with moxonidine at 60 and 120 μg/kg/h for 4 weeks, resulted in a mild additional decrease in the intensity of the 85-kDa bands to 51.1 ± 2.2 and 46.8 ± 3.3%, respectively (Fig. 4). A modest increase in the intensity of ~29-kDa band (data not shown) was observed in right atria of SHR (107 ± 2%) that decreased to 94 ± 1, 87 ± 3, and 83 ± 5% after 1 week of moxonidine at 10, 60, and 120 μg, respectively. In left ventricles, chronic treatment of SHR with moxonidine (120 μg) for 1 and 4 weeks did not alter the intensity of the bands corresponding to 85 kDa, but slightly decreased the 29-kDa band to 89 ± 3%, and after 4 weeks to 89 ± 1%.

Compared with corresponding WKY (considered as 100%), the percentage of increase in SHR right atrial B_{max} correlated with the percentage of increase in the density of the 85-kDa band ($R^2 = 0.7744$; $p < 0.03$), but not with the 29-kDa protein band. Moxonidine treatment resulted in a dose-dependent decrease in B_{max} and in the 85-kDa band compared with corresponding saline-vehicle treated SHR (considered as 100%). Figure 5 shows that the percentage of decrease in B_{max} correlated with the percentage of decrease in the 85-kDa band ($R^2 = 0.5700$; $p < 0.006$), but not with the 29-kDa band ($R^2 = 0.1754$; N.S.), suggesting that the 85-kDa protein may represent imidazoline I_1-receptors in the heart. A weak, but significant correlation was found between the 85- and the 29-kDa protein band ($R^2 = 0.2717$; $p < 0.03$), in moxonidine-treated SHR, implying that the two receptor proteins arise from the same gene.

Treatment of SHR with hydralazine for 1 week resulted in blood pressure reduction from 193 ± 8 to 135 ± 5 mm Hg ($p < 0.02$), whereas blood pressure remained in control rats at 186 ± 11 mm Hg. However, hydralazine treatment did not alter imidazoline receptor proteins measured by immunoblotting.

Discussion

The major findings of this study are as follows: 1) First time localization of α_2-adrenoceptors in heart atria and ventricles; and 2) demonstration that heart imidazoline I_1-receptors but not α_2-adrenoceptors are regulated in SHR, and in response to chronic in vivo exposure to a selective imidazoline receptor agonist, suggesting that heart I_1-receptors are subject to regulation. In addition, 3) the parallel change in receptor B_{max} and the 85-kDa imidazoline receptor protein, suggest that this protein may represent cardiac I_1-receptors.

Pharmacological and molecular cloning studies have revealed three α_2-adrenoceptor subtypes: α_{2A} (α_{2D} in rats), α_{2B}, and α_{2C} (Link et al., 1996; Altman et al., 1999). In the human heart, mRNA for all three α_2-adrenoceptors subtypes have been detected by PCR (Brodde and Michel, 1999). However, probably due to very low expression relative to α_1- and β_1-adrenoceptors, previous studies have not been successful in demonstrating α_2-adrenoceptors in the heart at the protein level through radioligand binding studies (Brodde and Michel, 1999). In the present study, demonstration of α_2-adrenoceptors in the heart was achieved by multiple approaches. Quantitative receptor autoradiography was used in conditions where binding of ^{125}I-PIC to adrenoceptors versus I_1-receptors was optimized by using high $MgCl_2$ (10 mM) concentration in the incubation buffer (Ernsberger et al., 1995). Furthermore, because radioligands cannot fully discriminate between α_2-adrenoceptor subtypes, further identification was obtained by immunoblots and RT-PCR, using specific rabbit polyclonal antibodies and primers for each subtype (Zou and Cowley, 2000). However, cellular localization of these receptors needs further experiments, because receptor subtypes were detected in whole cardiac tissue, which involves several cell types, including fibroblasts and myocytes, myocardial blood vessels, nerve terminals and intracardiac neurons (Armour, 1999).

TABLE 2
Kinetic parameters of cardiac I_1-receptors

	SD	WKY	SHR			
Moxonidine (μg/kg/h)	0	0	0	10	60	120
Right atria						
B_{max} (fmol/unit area)	18.3 ± 1.4	21.8 ± 1.4	40.0 ± 2.9*	18.2 ± 0.4**	10.2 ± 1.7**	7.0 ± 0.6**
K_d (nM)	3.9 ± 0.7	4.8 ± 0.4	5.8 ± 0.9	3.7 ± 1.2	5.1 ± 0.6	4.8 ± 0.5
Left atria						
B_{max} (fmol/unit area)	16.0 ± 2.6	12.2 ± 1.1	27.7 ± 2.8*	12.3 ± 0.6**	7.9 ± 1.1**	7.1 ± 0.4**
K_d (nM)	4.3 ± 0.8	4.5 ± 0.5	5.8 ± 0.9	2.1 ± 0.8	3.8 ± 0.4	4.8 ± 0.1
Right ventricles						
B_{max} (fmol/unit area)		5.1 ± 0.5	6.4 ± 0.5	4.1 ± 0.4	7.3 ± 0.4	8.1 ± 1.2
K_d (nM)		1.3 ± 0.1	0.8 ± 0.3	0.4 ± 0.1	1.0 ± 0.1	0.9 ± 0.2
Left ventricles						
B_{max} (fmol/unit area)		13.1 ± 2.5	10.5±0.8		11.7 ± 0.6	10.3 ± 0.7
K_d (nM)		3.7 ± 0.6	4.1 ± 0.7		3.9 ± 0.4	3.6 ± 0.3

*$p < 0.01$ versus WKY.
**$p < 0.01$ versus vehicle-treated SHR.

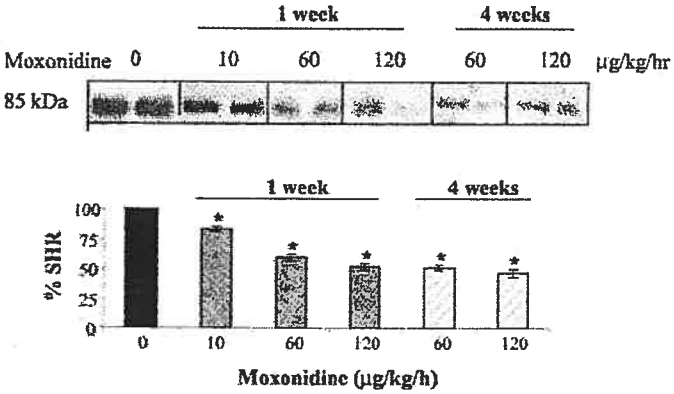

Fig. 4. Representative immunoblot and densitometric measurement of the 85-kDa imidazoline receptor protein in right atria of SHR after treatment with moxonidine (0, 10, 60, and 120 μg/kg/h) for 1 week and 60 and 120 μg/kg/h for 4 weeks. Data normalized to corresponding β-actin are presented as percentage of change from vehicle-treated SHR (considered as 100%). *, $p < 0.01$ versus vehicle-treated SHR.

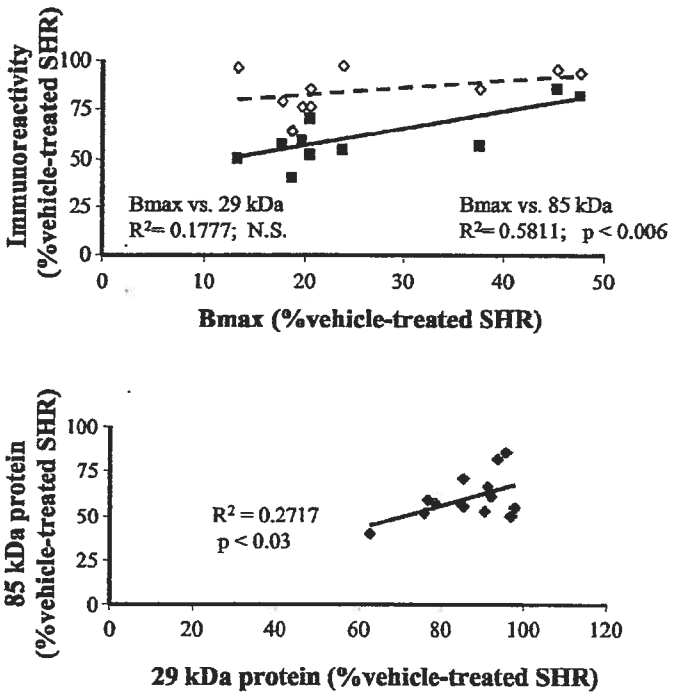

Fig. 5. Top, correlation between percentage of decrease in moxonidine-treated SHR (vehicle-treated SHR considered as 100%) right atrial B_{max} obtained from competitive binding assays versus percent decrease in the density of the 29-kDa (dotted line) and 85-kDa (solid line) bands obtained by immunoblotting. Bottom, correlation between percentage of change in the density of right atrial 85- versus 29-kDa bands obtained by immunoblotting.

The α_{2A}, abundant in the central nervous system, mainly in brain stem, is directly involved in regulating sympathetic outflow and seems to be the major presynaptic autoinhibitory receptor subtype (Altman et al., 1999). The α_{2B} is more abundant in arterial vascular smooth muscle cells and mostly responsible for vasoconstriction, and it is responsive to altered salt handling. The function of α_{2C} is not yet clear, but it may be the presynaptic autoreceptor in human atria (Rump et al., 1995; Hein, 2001).

The physiological significance of α_2-adrenoceptors in various heart chambers is beyond the scope of the present study. This study, however, provides strong evidence that α_2-adrenoceptors are present, albeit at low levels, in the rat heart atria and ventricles, at the levels of synthesis, protein expression, and binding activity, but these receptors seem not to be regulated by moxonidine, a selective agonist of imidazoline I_1-receptors. Because brain α_2-adrenoceptors have been shown to be selectively down-regulated in response to α_2-adrenoceptor agonists (Yakubu et al., 1990), and kidney imidazoline receptors to be down-regulated in response to imidazoline receptor agonists (Hamilton et al., 1993), the present findings imply that α_2-adrenoceptors in the heart interact weakly with moxonidine.

Most importantly, the present study confirms our previous finding that I_1-receptors are present in the heart and extend to demonstrate that up-regulated atrial I_1-receptors in SHR (El-Ayoubi et al., 2002a) are normalized by chronic in vivo exposure to I_1-receptor agonist. These receptors seem to be unrelated to imidazoline I_2-receptors, previously identified in the heart (Molderings and Gothert, 1999), because, by definition, the ligands used in the present study (^{125}I-PIC and moxonidine) show very low affinity to I_2-receptors (Bousquet, 1997).

Immunoblotting of heart membranes showed multiple molecular mass imidazoline receptor proteins similar to those so far described in brain and heart (El-Ayoubi et al., 2002a). Levels of 85-kDa proteins were increased in atria of untreated SHR compared with normotensive rats. Chronic moxonidine treatment, for short and long duration, was associated with decreased density of the 85-kDa bands in SHR atria. It is interesting to note that atrial 85-kDa but not the

29-kDa imidazoline receptor proteins vary in parallel to values of B_{max} for I_1-sites determined by ^{125}I-PIC binding. This correlation leads us to propose that the 85-kDa protein may represent I_1-receptors in the heart, as has been suggested by Ivanov et al. (1998). Furthermore, the positive correlation between changes in the two receptor proteins implies that they may arise from the same gene.

Regulation of imidazoline receptors has been previously reported in other tissues and under different physiological and pharmacological manipulations, usually in a manner distinct from α_2-adrenoceptors. Chronic treatment with the prototypic antidepressant imipramine, down-regulates I_1-receptors in rat brainstem, without affecting α_2-adrenoceptors (Zhu et al., 1997). Also, renal I_1-receptors are up-regulated by subpressor doses of angiotensin II infusion (Ernsberger et al., 1991), and in kidneys of SHR (El-Ayoubi et al., 2002b), whereas α_2-adrenoceptors are either unchanged or decreased in 1K1C rat kidneys (Li et al., 1994).

In the present study, imidazoline receptors were not different in hearts of two normotensive strains, WKY and SD, but up-regulated in SHR hearts, and then normalized and down-regulated after rat treatment with moxonidine, for short or long duration. The mechanisms involved in receptor up-regulation may include sympathetic overactivity, elevated blood pressure, increased cardiac mass, and activated intracardiac neurohormones, such as angiotensin II and norepinephrine. Treatment with moxonidine inhibits or counteracts these effects and eventually may indirectly lead to down-regulation of its receptor. We have previously shown that moxonidine dose dependently reduced blood pressure in SHR (Menaouar et al., 2002). However, the dose of 10 μg of moxonidine, which had no effect on blood pressure in those rats (Menaouar et al., 2002), significantly reduced I_1-receptor protein and B_{max}. Furthermore, treatment of SHR with hydralazine, a vasodilator antihypertensive compound that reduced blood pressure to a similar magnitude achieved by 120 μg of moxonidine, had no effect on imidazoline receptor proteins. Further studies are needed to clarify the mechanisms involved, but the present results argue against receptor down-regulation occurring in response to reduction in blood pressure per se, and in favor of a direct effect of the ligand on the receptor. Other investigators demonstrated that stimulation of I_1-receptor with moxonidine leads to activation of PC-PLC and generation of diacylglycerol, which activates several isoforms of protein kinase C (Ernsberger, 1999). Protein kinase C results in functional desensitization of the I_1-receptor through phosphorylation of serine and threonine residues in the receptor intracellular loop (Eason and Liggett, 1996).

In conclusion, this study demonstrates that heart I_1-receptors but not α_2-adrenoceptors are up-regulated in SHR and normalized by chronic antihypertensive treatment with moxonidine. Cardiac I_1-receptor normalization occurred after 1 week of treatment, the time point when moxonidine resulted in reversal of left ventricular hypertrophy in these rats (Menaouar et al., 2002). Also, the presence of I_1-receptors in atria, tissues known to secrete or respond to natriuretic peptides, atrial natriuretic peptide and brain natriuretic peptide, suggest a functional relationship between the two systems. Therefore, heart I_1-receptors are subject to regulation by the cardiovascular environment. Future antihypertensive treatment with imidazoline drugs should consider the heart as a major target organ.

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