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BRADYKININ METABOLISM IN THE INFARCTED AND HYPERTROPHIED RAT HEART: COMPARATIVE EFFECT OF THE VASOPEPTIDASE INHIBITOR OMAPATRILAT VERSUS AN ACE INHIBITOR

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Summary

This study had a two-fold purpose. First, having previously characterized the metabolism of BK inside the normal heart, we now wanted to define the influence that myocardial infarction and the ensuing left ventricular hypertrophy would have on this same metabolism. The effect of myocardial infarction and left ventricular hypertrophy on the metabolism of BK is particularly interesting, since accumulation of BK most likely plays a role in the cardioprotective effects of ACE inhibitors, a class of therapeutic agents successfully used in the treatment of these heart conditions. The second purpose of our study was to define the participation of ACE and that of NEP to the metabolism of BK inside the heart. These results would not only support the participation of BK in the beneficial effects of ACE inhibitors, but they would also explain the enhanced cardioprotective effect that dual vasopeptidase inhibitors, which act by simultaneously inhibiting both ACE and NEP, have over simple ACE inhibitors.

In order to study the influence of myocardial infarction and left ventricular hypertrophy on the cardiac metabolism of BK, myocardial infarction was induced in rats by left anterior descendant coronary artery ligature. The animals were then sacrificed and their hearts sampled, respectively 1, 4 and 35 days following infarction. BK metabolism was assessed by incubating synthetic BK with cardiac membranes from sham hearts, and infarcted (scar) and non-infarcted regions of infarcted hearts. A highly specific enzyme immunoassay developed in our laboratory was used to quantify the residual BK during incubation and allowed us to evaluate the half-life of BK. This half-life showed significant differences among the 3 types of tissues at 4 days [sham heart (114 ± 7 sec) > non-infarcted region (85 ± 4 sec) > infarcted region (28 ± 2 sec)] and 35 days post-MI [sham heart (143 ± 6 sec) = non-infarcted region (137 ± 9 sec) > infarcted region (55 ± 4 sec)]. No difference was observed however one day post-myocardial infarction. The participation of ACE and NEP in the metabolism of BK was defined by preincubation of the membrane preparations with enalaprilat, an ACE inhibitor, or omapatrilat, a dual vasopeptidase inhibitor. Enalaprilat significantly prevented the rapid degradation of BK in every tissue type and at

every sampling time. Moreover, omapatrilat significantly increased the half-life of BK, when compared to enalaprilat, in every tissue type and at every sampling time. The participation of ACE and NEP in the cardiac metabolism of BK was confirmed by quantifying the activity of each enzyme using synthetic substrates and by localizing both enzymes in the myocardium with highly specific antibodies.

Our results demonstrate, for the first time, that experimental myocardial infarction followed by left ventricular dysfunction significantly modifies the metabolism of exogenous BK by heart membranes. ACE and NEP participate in the degradation of BK since both enalaprilat and omapatrilat have potentiating effects on the half-life of BK.

Résumé

Les kinines sont de puissantes substances autacoides mieux connues pour leur actions pro-inflammatoires et vasodepressives. Dans le système cardiovasculaire, les kinines jouent également un rôle cardioprotecteur et vasoprotecteur en stabilisant le métabolisme cellulaire, réduisant ainsi la prolifération et la mort cellulaire en réponse à des conditions adverses telles l'ischémie. Les tissus comme le coeur contiennent toutes les substances nécessaires à la production et à la dégradation des kinines. Les niveaux tissulaires de kinines sont en effet finement ajustés aux besoins métaboliques de chaque tissu.

Suivant l'infarctus du myocarde, la capacité du ventricule à pomper le sang est réduite, entrainant une diminution du débit cardiaque. Le système cardiovasculaire répond par une série de changements physiologiques visant à rétablir le débit cardiaque à des niveaux correspondant aux besoins métaboliques de l'organisme. Le coeur subit un remodelage impliquant hypertrophie, fibrose et apoptose, et la post-charge est augmentée par une intensification du tonus vasculaire et par un acroissement de la rétention hydro-sodée. Bien que ces réponses du système cardiovasculaire réussissent à rétablir le débit cardiaque peu après l'infarctus, elles s'avèrent être néfastes à long terme, car elles accélèrent la dégénérescence et la mort des cellules survivantes et conduisent le coeur vers l'insuffisance cardiaque. L'administration d'Inhibiteurs de l'Enzyme de Conversion de l'Angiotensine (IECA) après l'infarctus du myocarde permet de ralentir, voire même renverser, le remodelage cardiaque et de diminuer considérablement la mortalité et la morbidité suivant l'infarctus. Cet effet bénéfique des IECA serait dû d'une part à la réduction de la formation d'angiotensine II et d'autre part à l'accumulation de bradykinine (BK). Ces deux substances ont des effets inverses sur le système cardiovasculaire. L'angiotensine II stimule le remodelage cardiaque et augmente la pression artérielle ainsi que la rétention hydro-sodée, alors que la BK exerce des effets stabilisateurs sur le muscle cardiaque et réduit la pression artérielle.

Dans ce projet, nous nous proposons d'étudier le métabolisme de la BK au niveau du tissu cardiaque après l'infarctus du myocarde et pendant le développement de

l'insuffisance cardique qui s'en suit. Le métabolisme de cet autocoïde cardioprotecteur qu'est la BK à déja été caractérisé dans le coeur normal, mais pas encore dans des circonstances pathologiques où la BK pourrait avoir un effet bénéfique. Dans le coeur sain, l'ECA s'est avérée être responsable de la majorité du métabolisme de la BK, alors que d'autres enzymes, telles l'endopeptidase neutre 24.11 ou les carboxypeptidases, ne jouent qu'un rôle mineur, qui peut cependant devenir important si l'ECA est inhibée. Afin d'élucider le mécanisme d'action des IECA, nous nous proposons également d'étudier leur effet sur le métabolisme tissulaire cardiaque de la BK, après l'infarctus du myocarde et lors du développement de l'insufisance cardiaque, soit au moment-même où ces IECA exercent leur effets cardioprotecteurs. Une nouvelle classe d'agents pharmacologiques inhibant à la fois l'ECA et l'endopeptidase neutre 24.11 a été mise au point pour ajouter aux effets bénéfiques des IECA, celui de l'accumulation des peptides natriurétiques, principalement métabolisés par l'endopeptidase neutre 24.11. Ces inhibiteurs mixtes ont démontré jusqu'à date des effets cardioprotecteurs supérieurs à ceux des IECA. La BK joue vraisemblablement un rôle dans l'effet cardioprotecteur de ces inhibiteurs mixtes, étant donné que l'endopeptidase 24.11 est impliqué dans son métabolisme, et ce surtout lors de l'inhibition de l'ECA. Pour éclaircir ce rôle de la BK, nous voulons également comparer dans le cadre de cette étude l'effet d'un inhibiteur mixte à celui d'un IECA, sur le métabolisme de la BK dans les circonstances décrites plus haut.

Pour étudier le métabolisme de la BK après l'infarctus du myocarde, nous avons utilisé un modèle animal mis au point par l'équipe de Pfeffer et al.. Un infarctus du myocarde a été induit chez le rat par ligature de l'artère coronaire antérieure gauche descendante. Les animaux ont été sacrifiés 1, 4 ou 35 jours suivant cette chirurgie et leur coeurs excisés. Les pressions intraventriculaires ainsi qu'un electrocardiogramme ont été obtenus avant le sacrifice. Les coeurs ont été classifiés selon l'electrocardiogramme en deux groupes: d'un côté les coeurs infarcis ayant un infarctus moyen ou grand, et de l'autre les coeurs sham présentant un petit infarctus ou aucun infarctus. Suite à leur excision, les coeurs ont été perfusés avec une solution saline et les coeurs infarcis ont été séparés en deux morceaux: le morceau infarci ou cicatrice de la paroi ventriculaire gauche et le reste du

coeur, ou morceau non-infarci. Pour étudier le métabolisme de la BK par les enzymes situées sur les membranes cellulaires cardiaques des morceaux de coeurs infarcis ou sham, nous avons utilisé un modèle *in vitro* précédemment employé par Blais et al. dans l'étude du métabolisme de la BK par le coeur normal. Les morceaux de tissu cardiaque ont été homogénéisés et les membranes ont été séparées du cytosol par ultracentrifugation, puis resuspendues dans une solution tampon Tris-HCl. Nous avons incubé de la BK synthétique (471 nM) dans ces suspensions membranaires, à 37 °C, en présence ou en absence d'inhibiteur enzymatique. Les inhibiteurs utilisés ont été soit l'IECA enalaprilate (130 nM) ou l'inhibiteur mixte omapatrilate (510 nM). Le métabolisme de la BK a été arrêté en précipitant les protéines du milieu d'incubation par l'ajout d'éthanol froid à différents moments suivant l'ajout de BK. Une méthode de dosage immunologique hautement spécifique developée au laboratoire a été utilisée pour mesurer la quantité de BK résiduelle dans les extraits éthanoliques. À l'aide de ces résultats nous avons déterminé la demi-vie de la BK dans les différents morceaux cardiaques (sham, infarcis ou non-infarcis; 1, 4 ou 35 jours post-infarctus) en présence ou en absence des inhibiteurs de l'ECA et/ou de l'endopeptidase neutre 24.11. Ceci nous a permis de calculer le pourcentage d'activité relative de l'ECA et de l'endopeptidase neutre 24.11 dans le métabolisme de la BK dans les différents milieux d'incubation. Afin de vérifier que la BK immunoréactive dosée dans les extraits éthanoliques correspond bien au peptide natif, nous avons séparé le contenu de ces extraits par HPLC et dosé les différentes fractions d'effluent par la même méthode immunologique. L'expression de l'ECA et de l'endopeptidase neutre au niveau tissulaire cardiaque ont été confirmées à l'aide d'une méthode immunohistochimique avec des anti-corps dirigés contre ces enzymes.

La présence d'un infarctus accélère significativement le métabolisme de la BK dans les coeurs infarcis, alors que la demi-vie de la BK demeure inchangée avec le temps dans les coeurs sham. Cette demi-vie est réduite considérablement entre 1 et 4 jours suivant l'infarctus lorsqu'on compare les coeurs infarcis aux coeurs sham. Alors que dans les morceaux infarcis cette réduction se maintient à 35 jours, le développement d'insuffisance cardiaque ramène la demi-vie de la BK dans les morceaux non-infarcis à

des valeurs correspondant aux coeurs sham. La présence d'enalaprilate ou d'omapatrilate prolonge significativement la demi-vie de la BK dans tous les types de tissu cardiaque et à tout moment suivant l'infarctus du myocarde. De plus, l'omapatrilate démontre un effet protecteur supérieur à celui de l'énalaprilate en prolongeant la demi-vie de la BK plus que ne le fait l'enalaprilate. Bien que l'effet des deux inhibiteurs soit présent dans tous les types de tissu et à tout moment, dans les morceaux non-infarcis il augmente entre un et 4 jours suivant l'infarctus, alors que dans les morceaux infarcis il n'augmente qu'entre 4 et 35 jours suivant l'infarctus. La participation relative de l'ECA au métabolisme de la BK demeure supérieure à celle de l'endopeptidase neutre 24.11 dans tous les types de tissu et à tout moment suivant l'infarctus. La participation relative de l'ECA augmente avec le temps dans les coeurs infarcis, et le fait d'avantage dans les morceaux infarcis que dans ceux non-infarcis. La participation de l'endopeptidase neutre 24.11 par contre diminue avec le temps dans les morceaux infarcis alors qu'elle reste inchangée dans les morceaux non-infarcis. Un jour après l'infarctus, la participation de l'endopeptidase neutre 24.11 est presque aussi importante que celle de l'ECA dans les morceaux infarcis. Ces résultats suggèrent que l'effet bénéfique des inhibiteurs mixtes se manifeste tôt après l'infarctus du myocarde. L'immunohistochimie n'a révélé la présence d'endopeptidase neutre 24.11 que dans les morceaux infarcis alors que la présence d'ECA s'est manifestée aussi dans les morceaux non-infarcis et dans les cours sham.

Les résultats de cette étude ont clairement démontré que le métabolisme tissulaire cardiaque de la BK est affecté par la présence de pathologies, telles l'infarctus du myocarde et le développement d'insuffisance cardiaque. La réduction de la demi-vie de la BK suivant l'infarctus pourrait être expliquée par la présence d'un foyer inflammatoire et par l'induction d'enzymes métabolisant la BK sur les cellules cardiaques. Nous avons également démontré que dans des circonstances pathologiques comme l'infarctus ou l'insuffisance cardiaque l'ECA demeure l'enzyme principal responsable du métabolisme de la BK au niveau tissulaire cardiaque. De plus, nos résultats ont démontré que l'omapatrilate exerce un effet protecteur de la BK supérieur à celui de l'énalaprilate. Ceci révèle l'importance de l'endopeptidase neutre 24.11 dans le métabolisme de la BK

endogène au niveau cardiaque et contribue à l'élucidation du mécanisme d'action cardioprotecteur des inhibiteurs mixtes tels l'omapatrilate.

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List of abbreviations

AC: adenylate cyclase
ACE: angiotensin I-converting enzyme, also known as kininase II
ACEi: angiotensin I-converting enzyme inhibitor(s)
ADH: anti-diuretic hormone, also known as arginine vasopressin
Ala: alanine
AMC: 7-Amido-4-methyl coumarin
AMP: adenosine monophosphate
Ang I, II, III and IV: angiotensin I, II, III and IV
ANP: atrial natriuretic peptides
Arg: arginine
AT_1: type-1 angiotensin receptor
AT_2: type-2 angiotensin receptor
ATP: adenosine triphosphate
AVP: arginine vasopressin, also known as anti-diuretic hormone
B_1: type-1 bradykinin receptor
B_2: type-2 bradykinin receptor
BK: bradykinin
BNP: brain natriuretic peptide
cAMP: cyclic adenosine monophosphate
cGMP: cyclic guanosine monophosphate
CGRP: calcitonin gene-related peptide
CHAPS: 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulphonic acid
cNOS: constitutive nitric oxide synthase
CNP: C-natriuretic peptide
COX: cyclooxygenase
CSF: cerebro-spinal fluid
DAG: diacylglycerol
des-Arg^9-BK: des-arg^9-bradykinin
DPhe: d-phenylalanine
ECG: electrocardiogram
EDHF: endothelium-derived hyperpolarizing factor
EGF: epithelial growth factor
EOX: epoxygenase
ER: endoplasmic reticulum
ET_A: type-A endothelin receptor
ET_B: type-B endothelin receptor
FXI: coagulaction factor XI
FXII: coagulation factor XII
GC: guanylate cyclase
GTP: guanosine triphosphate
HFBA: heptafluorobutyric anhydride
HMWK: high molecular weight kininogen
HPLC: high performance liquid chromatography

IC_{50}: inhibitory concentration at which 50% of the enzyme activity is inhibited
IgG: immuno-globulin G
IgM: immuno-globulin M
IL-1 (IL-1α, IL-1β): interleukin-1 (interleukin-1 alpha, interleukin-1 beta)
IL-2: interleukin-2
IL-6: interleukin-6
IL-8: interleukin-8
iNOS: inducible nitric oxyde synthase
IP_3: inositol 1,4,5-triphosphate
K_i: inhibitory constant
K_m: Michaelis-Menten constant
K_{cat}: catalytic constant
LHRH: luteinizing hormone-releasing hormone
LMWK: low molecular weight kininogen
LPS: lipopolysaccharides
LVDP: left ventricular end-diastolic pressure
LVH: left ventricular hypertrophy
MI: myocardial infarction
MDP: muramyl dipeptide
mRNA: messenger ribonucleic acid
n: number of subjects
NE: norepinephrine
NEP: neutral endopeptidase, E.C. 24.11, also known as neprilysin
NEPi: neutral endopeptidase, E.C. 24.11 inhibitor(s)
NO: nitric oxide
NOS: nitric-oxide synthase
P: probability that a statistical relationship may be due to a random error
PAF: platelet aggregating factor
PGE_2: prostaglandin E_2
PGI_2: prostacyclin
PI: prostacyclin receptor
PKC: protein kinase C
PLA_2: phospholipase A_2
PLC: phospholipase C
PLD: phospholipase D
PRA: plasma renin activity
RAS: renin-angiotensin system
RNA: ribonucleic acid
SEM: standard error of the mean
$t_{1/2}$: half-life
TNFα: tumor necrosis factor alpha
V_1: type-1 arginine vasopressin receptor
V_2: type-2 arginine vasopressin receptor

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Chapter 1:

INTRODUCTION

1. Kinins – origin, nature, properties and metabolism

Kinins are naturally occurring peptides composed of 8 to 11 amino acids. They are better known for their pro-inflammatory activity and for the role they play in acute and chronic inflammatory reactions. Cardiovascular pathological conditions such as myocardial infarction and left ventricular hypertrophy involve inflammatory mediators such as kinins. Pharmacological agents used in this setting rely in part on the cardioprotective effects of kinins to prevent or reverse the pathological changes undergone by the heart. The present study aims at elucidating the cardiac metabolism of kinins in order to better understand their cardioprotective role in the development of heart failure.

Kinins are produced in the circulating blood or locally in tissues by a limited enzymatic hydrolysis of their precursors, the kininogens. Following their synthesis, kinins are rapidly inactivated by numerous enzymes, the kininases, which are present everywhere in the body. Their short half-life and the ubiquitous distribution of the inactivating enzymes results in a local activity of the kinins at their site of release from kininogens. Kinins are thus considered as paracrine or autocrine substances and not endocrine ones.

Kinins act by binding to two types of receptors: the B_1 and the B_2 receptors. These G-protein coupled receptors are located on the cellular cytoplasmic membrane of several different cell types, themselves spread throughout the entire body. Kinins thus have a wide range of activities, the result of their action depending on the stimulated cell and receptor types. Their major effects are summarized below:

1. Vascular smooth muscle relaxation and vasodilatation;
2. Smooth muscle contraction in the gut and airways;
3. Increase of vascular permeability leading to edema;
4. Liberation of multiple inflammatory mediators;
5. Stimulation of sensory neurons.

Within the cardiovascular system, the kinins exert vasodilator and anti-proliferative effects and are therefore considered as cardioprotective and vasoprotective substances.

Tissues such as the heart continuosly synthesize and release kallikrein, kininogen and kinins at levels finely tuned to their local metabolic demands.

1.1. The kininogen-kallikrein-kinin system

The precursors of kinins are larger proteins called kininogens. Kinins are released from kininogens by the hydrolytic action of many specific and non-specific enzymes. The enzymes predominantly responsible for this hydrolysis are the kallikreins. This substrate-enzyme-product system is called the kininogen-kallikrein-kinin system. It is involved mainly in inflammation and blood clotting.

1.1.1. The kininogens

To date, three types of kininogens have been identified in mammals. These three types of kininogens are high molecular weight kininogen (HMWK), low molecular weight kininogen (LMWK) and T-kininogen. The third type, T-kininogen, is only present in rats.

All three types of kininogens originate in the liver and result from the alternative splicing of the same gene. They are glycoproteins (α_2-globulins) composed of two chains, a heavy one and a light one, bound together by a disulfide bond and by a kinin-containing domain. In addition to providing kinins, kininogens have other biological activities, owed to their chains. All three types of kininogens share homologous heavy chains that control inflammation by inhibiting cysteine proteases. The light chains are dissimilar among the three types. Only the function of the HMWK light chain is well known. It participates in the surface-dependent activation of the clotting cascade, by presenting to FXII two of its substrates, namely FXI and prokallikrein, for activation. (Müller-Esterl et al.., 1986; DeLa Cadena and Colman, 1991).

HMWK, LMWK and T-kininogen are synthesized mainly by hepatocytes and then secreted into the circulation (Borges and Gordon, 1976; Kitamura et al.., 1985). The presence of HMWK or LMWK in endothelial cells, cardiac myocytes, vascular smooth

muscle cells, neutrophils and kidney cells, suggests however that kininogens are also locally synthesized in tissues (Schmaier et al., 1988; Iwai et al., 1988; Figueroa et al., 1988 and 1992; Yayama et al., 1998). Fibroblasts and tissues such as the kidneys, the brain or the lungs are known to produce T-kininogen (Marks and al., 1988; Mann and Lingrel, 1991; Takano et al., 1995). Moreover, kininogens themselves can bind to the plasma membrane of cells featuring specific receptors. Some cells such as endothelial cells, platelets and neutrophils are also able to internalize kininogens after binding them (Gustafson et al., 1986 and 1989; van Iwaarden et al., 1989). These observations strongly suggest that kininogens may be active and release kinins outside the circulation, within the tissues. The abundance of kininogens in the blood and within the vascular wall suggests that kininogenase activities are the rate-limiting step in the accumulation of kinins in tissues.

1.1.2. The kallikreins

Although several enzymes can release kinins from kininogens, kallikreins principally assume this role. Kallikreins are serine proteases having different origins, locations and functions. They are classified according to their origin either as tissue or plasma kallikreins. There may be a third type in rats, T-kininogenase, as neither plasma nor tissue kallikreins seem to be responsible for the release of T-kinin from T-kininogen *in vitro*.

Plasma kallikrein is encoded by a single gene and synthesized in the liver. Hepatocytes release prokallikrein into the bloodstream, as an inactive zymogen that circulates as a complex together with HMWK (Mandle et al., 1976). Trypsin, chymotrypsin and clotting molecules such as active Factor XII and thrombin can convert prokallikrein into active kallikrein (Kaplan and Silverberg, 1987; Bhoola et al., 1992). Once activated, kallikrein releases BK from its host HMWK and remains bound to this HMWK afterwards. While bound to HMWK, kallikrein is less susceptible to inhibition by plasma protease inhibitors such as the C_1 esterase inhibitor (Schapira et al., 1981). Plasma kallikrein can also release BK from LMWK, but it has a much greater affinity for HMWK. In order to release BK

from LMWK plasma kallikrein requires the presence of a second enzyme, elastase (Sato and Nagasawa, 1988).

Tissue kallikreins are synthesized mainly by the pancreas, brain, kidney, gut and some exocrine glands such as the sub-maxillary and sudoriferous glands, and they are present in a variety of body fluids, namely, saliva, urine, bile and plasma (reviewed by Bhoola et al., 1992). The cardiac tissue also expresses kallikein mRNA and protein (Nolly et al., 1992, 1994). Unlike plasma kallikrein, which is coded by a single gene, tissue kallikreins are coded by multiple genes (Macdonald et al., 1988). Tissue kallikreins act on LMWK to release kallidin in humans and BK in rats. They may also release BK from HMWK but with a lesser affinity than for LMWK. The abundance of kininogens in blood and within tissues suggests that kallikrein activity is the rate-limiting step in the accumulation of kinins in tissues. While their primary physiological function is considered to be the release of kinins from kininogens, tissue kallikreins may have a broader physiological role, as they can cleave other biologically important substrates such as proinsulin, angiotensinogen, the precursor of ANF or procollagenase (reviewed by Bhoola et al., 1992).

1.1.3. The kinins

As previously mentioned, kinins are naturally occurring peptides composed of 8 to 11 amino acids. Three kinins are present in mammals: bradykinin (BK), kallidin and T-kinin. The latter, T-kinin has only to been seen in rats. As will be discussed later, kinins are metabolized in a number of different ways. The metabolites resulting from the hydrolysis of the C-terminal Arginine are biologically active. These metabolites are desArg^9-bradykinin (desArg^9-BK), desArg^9-kallidin and desArg^9-T-kinin. Table I lists the amino-acid sequences of these six active peptides.

Bradykinin (BK)			Arg^1	Pro^2	Pro^3	Gly^4	Phe^5	Ser^6	Pro^7	Phe^8	Arg^9
Kallidin (Lys-BK)		Lys	Arg^1	Pro^2	Pro^3	Gly^4	Phe^5	Ser^6	Pro^7	Phe^8	Arg^9
T-kinin (Ile-Ser-BK)	Ile	Ser	Arg^1	Pro^2	Pro^3	Gly^4	Phe^5	Ser^6	Pro^7	Phe^8	Arg^9
DesArg^9-BK			Arg^1	Pro^2	Pro^3	Gly^4	Phe^5	Ser^6	Pro^7	Phe^8	
DesArg^9-kallidin (Lys-desArg^9-BK)		Lys	Arg^1	Pro^2	Pro^3	Gly^4	Phe^5	Ser^6	Pro^7	Phe^8	
DesArg9-T-kinin (Ile-Ser-desArg^9-BK)	Ile	Ser	Arg^1	Pro^2	Pro^3	Gly^4	Phe^5	Ser^6	Pro^7	Phe^8	

Table I: the amino-acid sequence of the six biologically active kinins: the three native peptides bradykinin, kallidin, T-kinin and their three respective des-Arg9-metabolites, des-Arg9-bradykinin, des-Arg9-kallidin and des-Arg9-T-kinin.

BK is the main native kinin within the bloodstream, where plasma kallikrein is pre-eminent and has a greater affinity for HMWK than for LMWK. Within tissues however, plasma kallikrein is less abundent, leaving tissue kallikreins responsible for most of the kinin-generating activity. Since tissue kallikreins have a greater affinity for LMWK than for HMWK, kallidin is believed to be the main native kinin inside the tissue compartment. Thus, the vascular compartment of the kininogen-kallikrein-kinin system generates mostly BK, while the tissue compartment generates mainly kallidin.

In mice and rats, tissue kallikreins are unable to generate kallidin (Lys-BK) from LMWK and instead generate BK, making it the main kinin in both the circulation and the tissues. The amino acid residue preceding BK in rodent LMWK is Arg. In human and bovine LMWK the amino acid preceding BK is Lys and cleavage by tissue kallikreins yields Lys-BK (Hess et al., 1996).

1.2. The metabolism of kinins

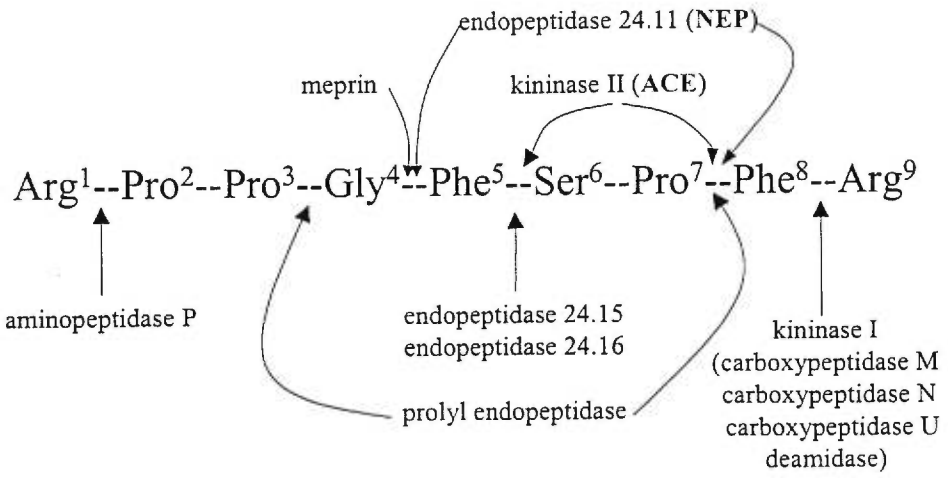

Figure 1: The Metabolism of Bradykinin
Peptide bonds in bradykinin cleaved by peptidases. Arrows show the primary site of cleavage, as established using purified enzymes and bradykinin in vitro. Adapted from Erdös and Skidgel, 1997.

A variety of enzymes may metabolize kinins *in vivo* and *in vitro*. These kininases belong mainly to families such as the zinc-metallopeptidases, but also to serine peptidases and proteinases, astacin-like metallopeptidases and cathepsins (Erdös and Skidgel, 1997). These enzymes have a ubiquitous distribution. They are present in most tissues such as the lungs, the kidneys, the brain or the heart and have various cellular localizations, such as on the plasma membrane, inside the cytosol or inside the organelles. Because of their wide distribution and high affinity for kinins (particularly the angiotensin 1-converting enzyme), the kininases rapidly metabolize kinins at their site of release from kininogens. The half-life of kinins is thus very short and their blood and tissue concentrations range between ng/ml and pg/ml (Bhoola et al., 1992). Within the plasma, for example, the kinin half-life ranges from 10 to 50 seconds depending on the animal species (Décarie et al., 1996). It is also noteworthy that none of the kininases are specific for kinins. An alteration of the kininase activity may therefore affect other systems besides the kinins

such as the renin-angiotensin system or atrial natriuretic peptides. Figure 1 shows for each enzyme its corresponding cleavage sites on BK. Of the numerous enzymes that cleave BK, the most important ones *in vivo* are probably angiotensin I-converting enzyme, neutral endo-peptidase 24.11, aminopeptidase P and carboxypeptidases N and M (Erdös and Skidgel, 1997).

1.2.1. Angiotensin I-converting enzyme (ACE, or kininase II)

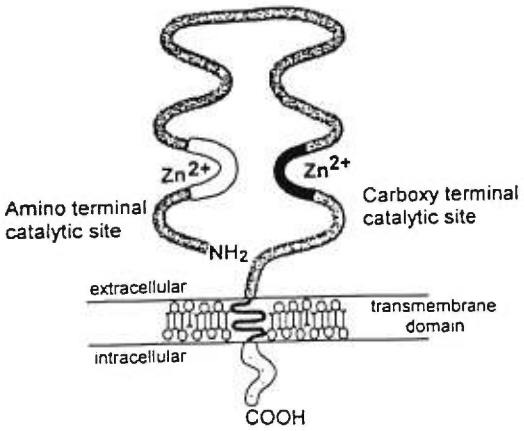

Figure 2: Angiotensin-converting enzyme (ACE) is a single chain glycoprotein of 140-170 kDa, composed of two domains, each containing an active site around a Zn^{++} cofactor. A hydrophobic anchor peptide inside the C-terminal domain tightly binds this metallopeptidase to the cytoplasmic membrane.
Structure adapted from Opie 1994.

Because of its high affinity for BK and its wide distribution in mammalian tissues, ACE is the predominant enzyme responsible for the degradation of BK in most organs. As shown in figure 2, ACE is a single chain glycoprotein of 140-170 kDa, composed of two domains, each with an active site containing a Zn^{++} cofactor. A transmembrane hydrophobic anchor peptide in the C-terminal domain tightly binds the metallopeptidase to the cytoplasmic membrane of a variety of cells (Weare et al., 1982; Alhenc-Gelas et al., 1990; Skidgel and Erdos, 1993). ACE is bound to endothelial cell membranes throughout the entire vascular system. The lungs, because of their heavy vascularisation, are particularly rich in ACE, which almost completely inactivates the circulating kinins during their first pass through the lungs (Erdös, 1990). The enzyme is also present on

subendothelial structures and especially on epithelial cells inside the kidney, intestine or choroid plexus, where ACE concentrations are higher than on the endothelium. (Erdös and Skidgel, 1997). Although it is mostly bound to plasma membranes, ACE is also released inside bodily fluids such as blood, urine or CSF, most likely by proteolytic cleavage of its anchor peptide (Erdös and Skidgel, 1989). Inside the heart, ACE is bound not only to the endocardium and coronary endothelium (Falkenhahn et al., 1995), but also, in lower concentrations, to the membranes of cardiomyocytes, fibroblasts and white blood cells (Yamada et al., 1991; Lindpainter and Ganten, 1991). ACE is not specific for BK, and its other substrates include des-Arg^9-BK, kallidin (Lys-BK), angiotensin I (Ang I), neurotensin, enkephalins, substance P and LHRH. *In vivo* however, there is only evidence for the hydrolysis of Ang I and BK (Corvol and Williams, 1997). The presence of a deletion allele in the ACE gene has been associated with a higher ACE activity in the serum, an increased conversion of Ang I to Ang II, an enhanced metabolism of BK and cardiovascular morbidity (Ueda et al., 1995; Brown et al., 1998). Because it has a higher affinity and turnover for BK (K_m=0.18μM) than for Ang I (K_m= 16μM), changes in ACE activity are more likely to affect BK than Ang I, whose product exerts pharmacological effects on the cardiovascular system opposite to those of BK. ACE acts by cleaving N-terminal dipeptides from its substrates. It converts the native BK_{1-9} into BK_{1-7} and BK_{1-7} into BK_{1-5}. (Erdös and Skidgel, 1997). ACE can also remove the N-terminal tripeptide of des-Arg^9-BK to yield BK_{1-5}. It has however less affinity for des-Arg^9-BK (K_m= 120 to 240μM) than for BK (K_m=0.18μM) (Inokuchi and Nagamatsu, 1981). Of the two domains of ACE, the C-terminal one is responsible for 76% of the BK degrading activity (Jaspard et al., 1993). Inhibitors and the medium's chlorine concentration influence the enzymatic activity and this influence is different from one domain to the other and from one substrate to another (Jaspard et al., 1993). On the plasma membrane, ACE is located in close proximity to the BK B_2 receptors. The ACE inhibitors potentiate the effects of BK not only by protecting BK from degradation, but also by acting on the BK receptors either directly or via an interaction with their neighboring ACE (Erdös and Skidgel, 1997).

1.2.2. Neutral Endopeptidase 24.11 (NEP or neprilysin)

NEP is a single chain transmembrane glycoprotein bound to the cytoplasmic membrane by an N-terminal peptide. It has a single active site, which contains the cofactor Zn^{++} and is therefore a member of the zinc metallopeptidases family. Like ACE, NEP has a wide tissular distribution and is present on the endothelium as well as on epithelial cells and other subendothelial structures, such as inside the respiratory tract, skeletal muscle, brain or kidneys. Unlike ACE however, its expression on the vascular endothelium is low and it is scarcely released inside the blood. Epithelia inside the kidneys, the placenta, the intestine and the choroid plexus are rich in NEP (Kerr and Kenny, 1974; Johnson et al., 1984; Turner, 1987; Ronco et al., 1988; Llorens-Cortes et al., 1992). Neutrophils express NEP, and elevated NEP levels are found on lymphocytes in certain blood disease states (Erdös et al., 1989; LeBien and McCormack, 1989). Although it is an integral membrane protein, the enzyme can also be released in bodily fluids. It has a very low concentration in plasma and a high concentration in urine, where it is responsible for most of the kininase activity. NEP cleaves peptides at the N-terminal of hydrophobic amino acids. Its *in vivo* substrates include BK, atrial natriuretic peptide (ANP), enkephalins, endothelins and substance P (Erdös and Skidgel, 1989; Scwartz et al., 1985; Vijayaraghavan et al., 1990). Like ACE, NEP cleaves BK at the Pro^7-Phe^8 bond. Prolonged incubation however, also results in the hydrolysis of the Gly^4-Phe^5 bond (Gafford et al., 1983). When compared to ACE, NEP has a lower affinity for BK (NEP: K_m=120μM vs. ACE: K_m=0.18μM) and thus plays a lesser role in the metabolism of this peptide. On the other hand, its similar distribution to ACE and its much higher turnover (lower K_{cat}) for BK make NEP an important metabolic pathway of BK when ACE is inhibited (Welches et al., 1993).

1.2.3. Aminopeptidase P

Unlike most aminopeptidases, aminopeptidase P, as its name implies, can remove the N-terminal amino acids from peptides containing Pro in the second position. Its active site contains a Zn^{++} cofactor. An N-terminal glycosylphosphatidylinositol anchor binds

Aminopeptidase P to plasma membranes and its proteolytic cleavage probably explains the presence of soluble aminopeptidase P in serum. A soluble form has also been identified in the cytosol. The cytosolic and the membrane-bound forms have different structures and substrate specificity and are most likely different gene products (Hooper, 1990; Turner and Hooper, 1997). Aminopeptidase P is not specific for BK. It can cleave most peptides and proteins such as BK and neuropeptide Y containing Pro in the second position. Many ACE inhibitors, which have a Pro-Pro like structure, fit inside the active site and inhibit aminopeptidase P by chelating its Zn^{++} cofactor (Hooper et al., 1992). This however is of little clinical consequence, since their IC_{50} for Aminopeptidase P is within the micromolar range, whereas that for ACE is within the nanomolar range. Aminopeptidase P-type activity has been detected in a variety of tissues. The membrane-bound form has been found in the lungs, kidneys and serum, whereas the cytosolic form has also been localized inside the brain, in erythrocytes and in platelets (Sidorowicz et al., 1984a, b; Hooper et al., 1990; Harbeck and Mentlein, 1991; Simmons and Orawski, 1992; Vanhoof et al., 1992; Orawski and Simmons, 1995). Aminopeptidase P may potentially play an important role in the pulmonary metabolism of BK, although it has a lower affinity for BK (K_m=21μM) than ACE (K_m=0.18μM) (Orawski and Simmons, 1995). Inside the rat lung, aminopeptidase P accounts for 75% of the BK degrading activity when ACE is inhibited, and together with ACE it is responsible for the entire metabolism (Ryan et al., 1994; Prechel et al., 1995). *In vivo* experiments have confirmed these results, but the higher levels of aminopeptidase P present in the rat lung relatively to the lungs of other species may reduce their relevance. Aminopeptidase P can also inactivate des-Arg9-BK (Simmons and Orawski, 1992; Orawski and Simmons, 1995). Its role in the metabolism of this kinin is perhaps greater than for BK, since ACE has a lower affinity for des-Arg^9-BK than for BK. In fact, the participation of kininases other than ACE in the hydrolysis of des-Arg^9-BK represents 65% in human, 48% in rabbit, 35% in dog and 95% in rat serum (Décarie et al., 1996).

1.2.4. Carboxypeptidase N

Carboxypeptidase N is a tetrameric enzyme of 270-330 kDa comprised of two heterodimers, each heterodimer containing one catalytic subunit and one glycosylated non-catalytic subunit stabilizing the catalytic one. The enzymatic hydrolysis requires the presence of Zn^{++} as a cofactor (Plummer and Hurwitz, 1978; Levin et al., 1982). Carboxypeptidase N is synthesized solely by the liver and released into the bloodstream. The enzyme is active only in circulation, since it is not stored in the liver and it is not subject to tissue uptake (Erdös and Skidgel, 1997). Carboxypeptidase N converts BK into its active metabolite des-Arg^9-BK and may cleave other substrates as well, such as anaphylatoxins, which have a C-terminal Arg or Lys (Skidgel, 1995). Although it is highly concentrated in the circulation, in most species the contribution of Carboxypeptidase N to the degradation of circulating BK is minor when compared to that of ACE (Erdös, 1979; Décarie et al., 1996). Carboxypeptidase N does however play an important role when ACE is inhibited (discussed by Erdös and Skidgel, 1997). Moreover, genetically low levels of Carboxypeptidase N, as well as its inhibition by protamine, were associated with undesirable systemic effects of BK (Matthews et al. 1980, 1986; Lowenstein et al. 1983; Morel et al. 1987; Lock and Hessel, 1990).

1.2.5. Carboxypeptidase M

Carboxypeptidase M is a single chain glycoprotein of 68 kDa, which is bound to the plasma membrane via a C-terminal glycosylphosphatidylinositol anchor. Like carboxypeptidase N, carboxypeptidase M removes the C-terminal Arg or Lys from a variety of peptides including BK and anaphylatoxins and Zn^{++} as a cofactor is required for enzymatic activity (Skidgel et al., 1989; Deddish et al., 1990). Carboxypeptidase M is present in many tissues, the lungs, kidney and placenta showing the highest levels and the blood vessels, intestine, brain and peripheral nerves showing lower levels (Turner and Hooper, 1997). A soluble form, probably released by proteolytic cleavage of the anchor, is found in various bodily fluids such as the urine, seminal fluid, amniotic fluid and bronchoalveolar lavage fluid (Skidgel et al., 1996). The enzyme is also expressed on

some white blood cells in amounts proportional to the differentiation of the cell. For example, macrophages express more carboxypetidase M than their precursor monocytes (Rehli et al., 1995). By converting BK into active des-Arg^9-BK, carboxypeptidase M modulates kinin activity outside the circulation, locally inside the tissues where it is present. Owing to its localization on white blood cells, this enzyme also plays an important role in local inflammation, by modulating the kinin activity and by degrading anaphylatoxins.

1.2.6. Carboxypeptidases of secondary importance

Carboxypeptidase U is a short-lived blood-borne enzyme, which is activated and inactivated during coagulation. The proenzyme is a glycoprotein synthesized in the liver. The active zinc metalloenzyme plays an important role in the fibrinolytic pathway and may convert BK into des-Arg^9-BK (Erdös and Skidgel, 1997). Because of its limited activity and its low affinity for BK (K_m=10000μM), it is unlikely that this enzyme plays an important role in the metabolism of BK, when compared with ACE (K_m=0.18μM), or in the production of circulating des-Arg^9-BK, when compared to carboxypeptidase N.

Deamidase (cathepsin A), a serine protease, is a dimeric glycoprotein localized inside lysosomes. Like the other carboxypeptidases it converts BK into des-Arg^9-BK. Its other substrates include substance P, neurokinin A, enkephalinamides, angiotensin I, chemotactic peptide and endothelin (Erdös and Skidgel, 1997). Deamidase has a ubiquitous distribution inside the body, the highest mRNA levels being found in the mouse kidney and placenta (Galjart et al., 1990). It is also highly active in macrophages, platelets, endothelial cells and fibroblasts. During inflammation, the enzyme is released from the lysosomes and has then access to extracellular kinins. Under normal conditions, deamidase may be involved in the recycling of the BK receptor by cleaving the receptor bound kinin after internalization of the receptor-ligand complex (Erdös and Skidgel, 1997).

Prolylcarboxypeptidase is a single chain glycoprotein, which like deamidase is a serine protease and is localized inside lysosomes. It removes the C-terminal amino acid from peptides having Pro in the penultimate position. Des-Arg^9-BK can thus be deactivated, but BK cannot be converted into des-Arg^9-BK. Prolylcarboxypeptidase has a ubiquitous distribution inside the body, with high mRNA levels inside the human placenta, lung and liver and a significant activity inside white blood cells, fibroblasts and endothelial cells (Kumamoto et al., 1981; Skidgel et al., 1981; Tan et al., 1993). Like deamidase, prolylcarboxypeptidase has access to extracellular des-Arg^9-BK upon its release from the lysosomes during inflamation. The enzyme may also be involved in the B_1 receptor recycling.

1.2.7. Endopeptidases of secondary importance

Endopeptidase 24.18 (meprin), a member of the astacin family of metalloproteases, is a membrane bound glycoprotein composed of two subunits. The α unit contains the active site and the β unit binds the enzyme to the membrane (Wolz and Bond, 1995). Endopeptidase 24.18 has very broad substrate specificity. Its *in vitro* substrates include BK, substance P, neuropeptide Y, parathyroid hormone, melanocyte stimulating hormone, neurotensin, LHRH and angiotensin I, II and III (Sterchi et al., 1997). It cleaves BK at the Gly^4-Phe^5 bond. Endopeptidase 24.18 seems to be confined to epithelial cells and has only been found in the intestine, kidney and salivary glands (Sterchi et al., 1988; Barnes et al., 1989; Craig et al., 1991). Its very low affinity for BK and its confined distribution make it unlikely that endopeptidase 24.18 plays a dominant role in degrading kinins.

Endopeptidase 24.15 is a single chain zinc-metallopeptidase with a ubiquitous distribution and a particularly high activity inside the brain and testis (Tisljar, 1993). Endopeptidase 24.15 is primarily a cytosolic enzyme, but around 20% of the enzyme in the rat brain is bound to membranes of subcellular structures (Acker et al., 1987). It is yet unclear whether the enzyme is bound to the external surface of the plasma membranes, thus questioning its role in the degradation of extracellular BK. *In vitro*, the enzyme

cleaves BK at the Phe^5-Ser^6 bond. Besides BK, neurotensin, substance P, LHRH and enkephalins precursors figure among its many substrates (Turner, 1997).

Endopeptidase 24.16 is very similar to endopeptidase 24.15. Both enzymes cleave BK at the same site *in vitro* and share the same distribution. The mainly cytosolic and mitochondrial localization of endopeptidase 24.16 does not exclude a small membrane-associated component, although evidence is lacking for an extracellular activity and particularly in the metabolism of BK (Erdös and Skidgel, 1997).

Prolylendopeptidase is another cytosolic enzyme found in most tissues with particularly high concentrations in the brain and kidneys (Wilk, 1983). It hydrolyses BK and des-Arg^9-BK *in vitro* at the Pro^7-Phe^8 bond and at the Pro^3-Gly^4 bond (Ward, 1991). This serine protease is not specific for kinins and can cleave a variety of other peptides containing Pro (Wilk, 1983). The cytosolic localization of this enzyme precludes it from playing a major role in the metabolism of kinins. However, like the other cytosolic enzymes it can participate in this metabolism if released in the extracellular medium by cell lysis and death.

1.2.8. BK metabolism in the heart

The nature of the enzymes that metabolize BK and their relative importance in this metabolism vary from one species to another and from one tissue to another. The metabolism of BK has been studied so far in different tissues, such as the kidney, the lung and skeletal muscle (Ura et al., 1987; Dragovic et al., 1993; Ward et al., 1995). In order to better elucidate the cardioprotective properties of BK, its metabolism inside the cardiovascular system has been adressed recently by work in our laboratory, whereby ACE has been shown to be the major BK-degrading enzyme in the serum as well as in the heart. In fact, depending on the species, ACE is responsible for 50% to 93% of BK metabolism in the serum (Décarie et al., 1996) and for 45% to 68% of the BK metabolism inside the heart tissue (Blais et al., 1997). Moreover, ACE has also been shown to be the major enzyme responsible for the degradation of BK at the coronary artery bed level

(Dumoulin et al., 1998). While such findings support a role for endogenous BK in the cardioprotective role of ACE inhibitor drugs (see section 3), they also emphasize other metabolic patways of BK as potential sites of action for cardioprotective agents. We have shown in our laboratory that the other BK-degrading enzymes besides ACE represent minor metabolic pathways inside the cardiovascular system in most species. The kininase I pathway (see figure 1, page 7) for instance, metabolizes only 2.6% of BK inside the human heart (Blais et al., 1997) and 3.4% of BK in the human serum (Décarie et al., 1996). Some of these minor metabolic pathways may however become important and physiologically relevant once ACE activity is inhibited, and particularly in a pathological setting. This is the case of the kininase I pathway during myocardial ischemia. Its product, des-Arg^9-BK, was not detected in the coronary effluent of normal rat hearts infused with BK, even in the presence of an ACE inhibitor (Dumoulin et al., 1998), but was released following reperfusion of the ischaemic rat heart (Lamontagne et al., 1995). Moreover, treatment with an ACE inhibitor enhanced this release of des-Arg^9-BK by the ischemic heart (Lamontagne et al., 1995). NEP may represent another minor metabolic pathway of BK which becomes relevant upon inhibition of ACE. At the coronary vascular bed level, NEP was shown to significantly contribute to the degradation of BK, but only when ACE activity was impaired (Dumoulin et al., 1998). In heart tissue, NEP may play an important role in regulating local kinin levels, as its inhibition affects myocardial perfusion by enhancing the effects of bradykinin (Piedimonte et al., 1994).

1.3. The bradykinin receptors

1.3.1. Agonists

Kinins exert their pharmacological effects via an interaction with cell surface bradykinin receptors. The existence of two different receptor subtypes, the B_1 and the B_2 receptors, was initially proposed based on their different responses to agonists (Regoli and Barabé, 1980). The cloning and expression of both B_1 and B_2 receptor subtypes and the development of selective antagonists later confirmed this classification. Bradykinin

receptors are classified as either B_1 or B_2 depending on the potency order of kinin receptor agonists:

B_1 receptors

Des-Arg^9-BK > Tyr(Me^8)-BK > BK

B_2 receptors

Tyr(Me^8)-BK > BK >> des-Arg^9-BK

The B_1 receptors respond preferentially to kinin metabolites lacking the C-terminal Arg (des-Arg^9-BK, des-Arg^9-kallidin or des-Arg^9-T-kinin) whereas the B_2 receptors respond only to native kinins. The presence of the C-terminal Arg is therefore essential for affinity for B_2 receptors and its removal, while canceling activity on these receptors, greatly enhances the affinity for B_1 receptors. The agonist order of potency of the naturally occurring kinins is listed in table II. These results were obtained in binding competition assays in human IMR-90 lung fibroblasts co-expressing both B_1 and B_2 receptors (Marceau, 1997).

Kinin compartment	*B_1 receptor*	*B_2 receptor*
Vascular	Des-Arg^9-BK (590) > BK (7800)	BK (2) >> des-Arg^9-BK (30000+)
Tissue	Des-Arg^9-kallidin (0.5) > kallidin (62)	Kallidin (7) >> des-Arg^9-kallidin (27000)
Both vascular and tissue	Des-Arg^9-kallidin > kallidin > des-Arg^9-BK > BK	BK ≥ kallidin >> des-Arg^9-kallidin ≥ des-Arg^9-BK

Table II: The agonist order of potency of the naturally occurring kinins. The values between parenthesis are affinity estimates: IC50 (nM) for B1 receptors and Ki (nM) for B2 receptors (table adapted from Marceau, 1997).

It is noteworthy that the B_1 receptors have more affinity for kallidin than for des-Arg9-BK, although kallidin is a native peptide containing the C-terminal Arg. This observation may be explained by an overestimation of the native kinin potency on B_1 receptors due to the kinin's partial conversion into its des-Arg^9 metabolite during the assay (discussed by Maceau and Regoli, 1991). However, the tissue kinin des-Arg^9-kallidin has a far greater affinity for B_1 receptors, than do the systemic kinins des-Arg^9-BK and BK, and may in fact be the physiological B_1 receptor ligand. This could indicate that B_1 receptors play a

greater role inside the tissues, than within the blood vessels. While the B_2 receptors respond similarly to tissue and systemic kinins and should thus be active at both tissue and vascular levels, the B_1 receptors may play a role exclusively within the tissue compartment of the kininogen-kallikrein-kinin system, which also includes the LMWK and the tissue kallikreins. (Hall, 1992; Hall and Morton, 1997; Hess, 1997)

1.3.2. Antagonists

The development of competitive B_2 receptor antagonists has taken place in three stages. Peptide analogs of BK provided the first generation of antagonists, of which [DPhe^7]-BK was the prototype (Vavrek and Stewart, 1985). Additional substitutions on [DPhe^7]-BK improved the affinity and selectivity of the antagonists, and the dimerization of some of these compounds further increased their affinity for the B_2 receptor. Drawbacks of this first generation of antagonists included a short half-life due to enzymatic degradation *in vivo*, a rather weak affinity when compared with BK, a limited selectivity and partial agonist activity inside certain tissues (Décarie, 1997). The replacement of amino-acids inside BK with artificial residues marked the next stage in the development of B_2 receptor antagonists. Hoe 140 was the prototype of the second generation of antagonists (Hock et al., 1991; Wirth et al., 1991). While Hoe 140 showed a remarkable selectivity for B_2 receptors, dimerization allowed for other compounds, such as CP0364 to act on both B_1 and B_2 receptors (Whalley et al., 1992). The use of artificial residues increased the affinity and metabolic stability of the second generation of antagonists, granting them a longer duration of action. Third generation B_2 receptor antagonists are entirely non-peptide compounds, some of them derived from plant extracts. While their affinity for B_2 receptors may be lower than second generation antagonists, they have an increased resistance to metabolism and a potential activity following oral administration, which was absent in previous generations. WIN64228 was the first relatively selective, high-affinity non-peptide B_2 antagonist (Salvino et al., 1993). Recently, other orally active compounds, such as FR173657 and FR165649, showed selective inhibition of B_2 receptors *in vivo* and *in vitro* (Inamura et al., 1997; Asano et al., 1998).

Development of B_1 receptor antagonists was based on modifications of the B_2 antagonists. The des-Arg^9-[Leu^8]-BK peptide, was the first B_1 antagonists and remained the most widely used in *in vivo* and *in vitro* studies, although additional substitutions or dimerizations further increased affinity for the B_1 receptor. Modifications of the second generation B_2 receptor antagonist Hoe 140 yielded the selective B_1 antagonist des-Arg^9-Hoe 140 sharing the high affinity and metabolic stability of its parent compound. Another promising artificially substituted peptide is R715, which proved to be partially resistant to ACE metabolism and to have a high affinity for the human and rabbit B_1 receptor. So far, non-peptide B_1 receptor antagonists have not yet been reported. (Hall and Morton, 1997; Marceau et al., 1998)

1.3.3. Expression and regulation

B_2 receptors have a ubiquitous distribution and have been identified in most species. They are present on neurons, endothelial cells, epithelial cells, hepatocytes, smooth muscle cells, fibroblasts, and possibly also leucocytes, such as macrophages and neutrophils. B_2 receptor-mediated effects have been demonstrated in the gastrointestinal tract, genitourinary system, respiratory tract, in neuronal tissue and in the cardiovascular system. B_2 receptors are constitutive and thus are present in tissues under normal, non-pathological conditions. While several compounds, such as cyclic adenosine monophosphate (cAMP), interleukin-6 (IL-6), interleukin-1 (IL-1), or tumor necrosis factor α (TNFα), may modulate the expression and synthesis of the B_2 receptors, they only modestly affect overall B_2 receptor activity, because the normal B_2 receptor levels are elevated. (Raymond et al., 1996; Hall and Morton 1997; Marceau, 1997).

B_1 receptors are not constitutive. In most species, including humans, they are absent from healthy tissues and are induced by noxious stimuli and disease. Some species however, such as the mouse, cat and dog, express B_1 receptors under normal conditions, owing probably to differences in the B_1 gene promoter region. The upregulation of B_1 receptors from their usual null levels has been evidenced in numerous *in vitro* and *in vivo* studies reviewed by Marceau F (1997). *In vivo*, in rats, rabbits or pigs, noxious stimuli such as

ultraviolet irradiation, the injection of lipopolysaccharides (LPS), mycobacterial products or cytokines, and diseases such as arthritis, genetic hypertension or septic peritonitis, induced responses to B_1 receptor agonists, ranging from hypotension to microvascular leakage and hyperalgesia. *In vitro*, induction of B_1 receptors was studied in various cell and tissue models, such as the rabbit aorta or human fibroblasts. IL-1 is the most potent stimulant of B_1 receptors, considerably amplifying the B_1 receptor mRNA content of human fibroblasts within 30 minutes of administration. Other B_1 stimulants include interleukin-2 (IL-2), interleukin-8 (IL-8), oncostatin M, LPS, muramyl dipeptide (MDP) and epithelial growth factor (EGF). Glucocorticosteroids suppress most of the IL-1, IL-2, LPS and MDP stimulated upregulation. The induction of B_1 agonist responses was also blocked by inhibiting either protein synthesis, RNA synthesis or protein translocation between the endoplasmic reticulum and the Golgi apparatus, strongly suggesting that B_1 receptors are synthesized *de novo* following proper stimulation. Their induction by cytokines liberated during the inflammatory response suggests that the physiological role of B_1 receptors may be limited primarily to mediating kinin responses in damaged tissues. (Marceau, 1997).

Responses to B_1 receptor agonists persist longer and are less subject to tachyphylaxis, than those elicited by B_2 receptor agonists. This difference may be explained by the rapid internalization of B_2 receptor-ligand complexes and the absence of internalization of B_1 receptor-ligand complexes. Although they are shorter acting, B_2 receptors mediate the majority of the effects of kinins *in vivo*, because of their abundance in most tissues under normal as well as pathological conditions. Inside damaged tissues however, the appearance of B_1 receptors following injury and disease significantly extends the intensity and the duration of action of kinins. In fact, the damaged tissues respond not only to native kinins via the B_2 receptors, but also to their des-Arg^9 metabolites via the B_1 receptors. Moreover, responses to kinins last longer inside those tissues, since B_1 active des-Arg^9 metabolites have longer half-lives than their parent kinins and since B_1 receptors are less prone to tachyphylaxis. (Marceau, 1997).

1.3.4. Molecular identification and cloning of B_1 and B_2 receptors

B_1 and B_2 receptors are products of different genes. The two receptor types are quite dissimilar, since they share only 36% of their amino-acid sequence in humans.

Human B_1 receptors were cloned inside *Xenopus laevis* oocytes by using mRNA from IL-1 stimulated human lung fibroblasts. The DNA sequence from the *Xenopus* oocytes encodes a G-protein coupled receptor of 353 amino acids having seven putative transmembrane domains. In humans, the B_1 receptor has a 36% amino acid sequence homology with the B_2 receptor and a 30% homology with the angiotensin II receptor. Across species, the B_1 receptors are homologues of the same gene. Slight differences in the amino-acid sequence from one species to another account for the different pharmacological profiles of receptors. Both human and rabbit (78% homology) B_1 receptors exhibit relatively high affinity for des-Arg^9-kallidin, but the human receptor has a seven-fold lower affinity for des-Arg^9-BK when compared to rabbit. This indicates that des-Arg^9-kallidin may be the more important ligand *in vivo*, since the evolutionary selective pressure to maintain affinity for des-Arg^9-kallidin was higher than that for des-Arg^9-BK. Although very similar (73% homology) to the human and rabbit receptors, the mouse B_1 receptor has more affinity for des-Arg^9-BK than for des-Arg^9-kallidin. This reversal of selectivity is probably due to the absence of kallidin in rodents, where tissue kallikreins generate BK instead of kallidin from LMWK. (Hess, 1997; Marceau, 1997).

B_2 receptors were cloned in various species, including the rat, mouse, human and rabbit. Their DNA sequence revealed a G-protein coupled receptor having seven transmembrane domains. Inside humans, the B_2 receptor is composed of 364 amino-acids. Directed mutagenesis experiments suggested that B_2 antagonists do not have the same binding site as BK on the B_2 receptor. Like B_1 receptors, B_2 receptors are slightly different from one species to another. Humans, rats and mice share 81% to 94% of the B_2 receptor amino-acid sequence. These differences in amino-acid sequences are responsible for the different pharmacological responses elicited across species. However, different pharmacological responses were noticed within the same species, suggesting the

existence of multiple B_2 receptor subtypes. Such was the case in the guinea pig, where B_2 antagonists acting on ileum preparations were inactive on trachea preparations. Moreover, in the rat vas deferens, B_2 receptors, according to their location on nerves or smooth muscle, reacted differently to both B_2 agonists and antagonists. This pharmacological evidence alone however, cannot sustain the existence of multiple B_2 receptor subtypes and additional studies involving cloning of the B_2 receptors and examination of B_2 receptor knock-out animals are needed to confirm their existence. So far, such additional studies suggest the existence of a single B_2 receptor subtype. B_2 receptor knock-out mice in which the gene encoding for the B_2 receptor was disrupted, lacked responsiveness to BK both in smooth muscle and neuronal preparations. The different pharmacological responses observed earlier could thus be explained by alternative splicing of the same gene and not by the co-existence of multiple receptor subtypes. (Décarie, 1997; Hess 1997; Hall and Morton, 1997).

1.3.5. Intracellular signaling pathways

Both B_1 and B_2 receptors are coupled to G proteins (see figure 3, page 23). The B_2 receptor activates phospholipase C (PLC), whose products include diacylglycerol (DAG) and IP_3. DAG activates protein kinase C (PKC), while IP_3 releases Ca^{++} from the endoplasmic reticulum. PKC activates phospholipase D (PLD) and inhibits production of cAMP by inhibiting adenylate cyclase (AC). The high levels of intracellular Ca^{++} directly induce contraction in smooth muscle cells and indirectly relax smooth muscle cells adjacent to the endothelium, via the nitric oxyde (NO) secondary mediator. Inside endothelial cells, the increased Ca^{++} levels activate the production of NO by the Ca^{++} dependent enzyme NO-synthase (NOS). NO, a short lived secondary mediator of BK, migrates out of the endothelial cell and into adjacent smooth muscle cells, where it induces relaxation and thereby vasodilatation. NO acts mainly through the generation of cyclic guanosine monophosphate (cGMP) and probably also by directly activating potassium channels. Phospholipase A_2 (PLA_2) is another Ca^{++} dependent enzyme activated by the high levels of intracellular Ca^{++} that follow BK receptor stimulation. PLA_2 is responsible for the production of various eicosanoids that act as secondary

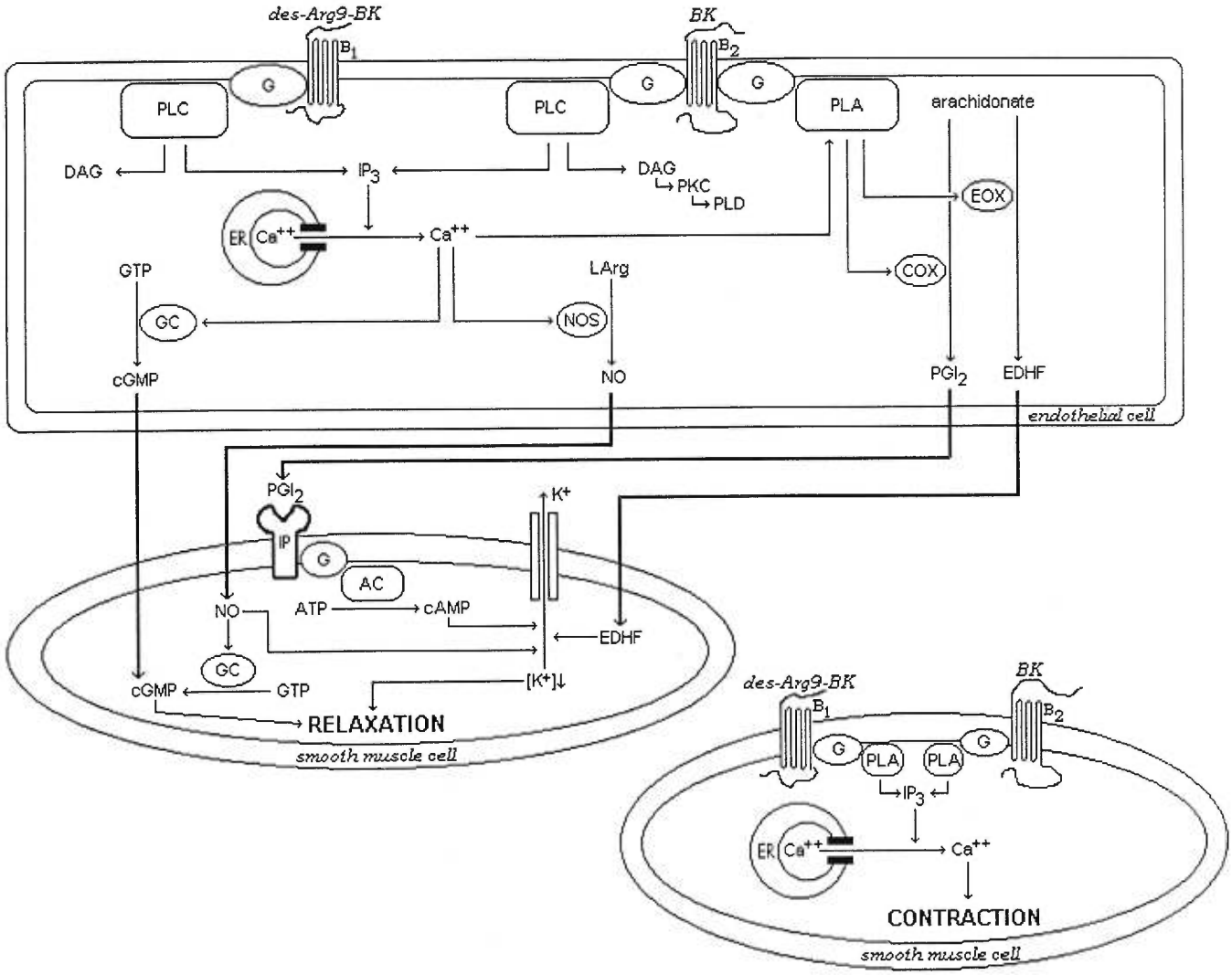

Figure 3: Intra-cellular pathways and secondary mediators mobilized following kinin receptor activation on endothelial cells and smooth muscle cells (figure adapted from Mombouli et al., 1996 and Décarie, 1997).

☞ B_1 and B_2: kinin receptors; PI: prostacyclin receptor; ER: endoplasmic reticulum; G: G protein; PLA, PLC and PLD: phospholipase A, C and D; AC: adenylate cyclase; GC: guanylate cyclase; NOS: nitric-oxyde synthase; COX: cyclooxygenase; EOX: epoxygenase; PKC protein kinase C; DAG: diacylglycerol; IP_3:inositol 1,4,5-triphosphate; GTP: guanosine triphosphate; cGMP: cyclic guanosine monophosphate; ATP: adenosine triphosphate; cAMP cyclic adenosine monophosphate; L-Arg: L-Arginine; NO: nitric oxide; EDHF: endothelium-derived hyperpolarizing factor; PGI_2: prostacyclin.

mediators of BK. Which particular eicosanoids are produced depends on the cell type stimulated by BK, the main ones being PGE_2, PGI_2, leucotrienes and platelet aggregating factor (PAF). Eicosanoids play an important role as secondary mediators, since inhibition of PLA_2 significantly reduces the effects of BK. cGMP is another secondary mediator of BK, which induces relaxation in smooth muscle cells. It is produced by an enzyme, guanylate cyclase, stimulated by the increased levels of intracellular Ca^{++}, eicosanoids or NO that follow BK receptor activation. PLA_2 in endothelial cells, when stimulated by BK, produces yet another vasodilatatory secondary mediator called endothelium-derived hyperpolarizing factor (EDHF), which induces relaxation of nearby smooth muscle cells by activating their potassium channels. (Bascands and Girolami, 1996; Décarie, 1997).

The B_1 receptor shares the same intracellular signaling pathways as the B_2 receptor. It increases production of DAG and IP_3 via activation of PLC. DAG is involved through its activation of PKC, in the weak mitogenic effect resulting from B_1 activation. IP_3, the other PLC product, releases Ca^{++} from intracellular stores. The increased cytosolic Ca^{++} levels are responsible for the smooth muscle contraction mediated by B_1 receptors in the rabbit aorta. Moreover, Ca^{++} dependent enzymes synthesize NO and eicosanoids, which are secondary mediators released under the action of the B_1 agonist des-Arg^9-BK. (Marceau, 1997).

1.4. The biological actions of bradykinin

1.4.1. Smooth muscle relaxation and contraction

Upon binding to receptors on smooth muscle cells, BK causes these cells to contract by increasing their cytoplasmic Ca^{++} concentration. This increase is due both to a rapid release of Ca^{++} from intracellular stores, and to a sustained entry of extracellular Ca^{++} through activated Ca^{++} channels. BK however, may also cause smooth muscle cells to relax. It does not so directly, by binding to receptors on the smooth muscle cells, but indirectly via mediators whose production and release it activates in nearby endothelial or neuronal cells. Myorelaxant mediators released from endothelial cells include NO,

EDHF, cGMP, PGI_2 and PGE_2 and those released from neuronal cells include substance P and CGRP. The BK-mediated bronchoconstriction in asthma and related inflammatory diseases of the airways is an example of a mixed direct and indirect response to BK. This smooth muscle cell contraction is elicited both via a direct increase of intracellular Ca^{++} and via the stimulation of adjacent nervous fibers releasing acetylcholine (Bhoola et al., 1992).

1.4.2. The cardiovascular system

Kinins are best known for their vasodilatory and hypotensive effects (Wirth et al., 1991). Genetically engineered rats in which the activity of the kinin-kallikrein system was augmented showed significant decreases in blood pressure, whereas B_2 receptor knock-out mice exhibited hypertensive phenotypes (Madeddu et al., 1997). In most species, intravenous administration of exogenous BK considerably lowers the blood pressure (Regoli and Barabé, 1980). This BK-mediated hypotension is abolished by administration of the B_2 antagonist Hoe 140 and is reversed by discontinuing the BK infusion (Wirth et al., 1991). Vasodilatation is the most likely explanation of this hypotensive effect of BK. It involves the production of vasodilatatory secondary mediators in the endothelium (NO, eicosanoids, EDHF) and their diffusion toward the vascular smooth muscle cells located inside the blood vessel wall (Lamontagne et al., 1992; Mombouli et al., 1996).

Although it is a potent vasodilator, BK may also constrict certain blood vessels as evidenced by its role during inflammation. BK is an important key-substance of the inflammatory process. It triggers the release of various cytokines and causes local edema. This latter effect is due to an increase of the vascular permeability and an increase of the intra-capillary pressure. BK contracts the capillary endothelial cells and thus enlarges the gaps that separate them (Gabbiani et al., 1970). It also increases the pressure inside capillaries by contracting the post-capillary veinules and dilating the pre-capillary arterioles (Hall, 1992).

Besides its vasomotor properties, BK also exerts protective effects on the cardiovascular system. It protects cells within this system against damages incurred by ischemia and prevents them from adversely proliferating in response to disease. These effects of BK may be explained by an enhancement of the cellular energy metabolism, which improves the stability and survival of the cell through adverse conditions. In fact, BK has been shown to potentiate glucose uptake, reduce lactate release and increase intracellular ATP, glutathione and creatinine contents, which are the cellular energy reserves. Prostacyclin likely mediates most of the protective metabolic effects of BK. The stabilizing effects of BK on the cardiovascular system also translate into a reduction of the incidence and duration of cardiac arrhythmias following myocardial ischemia and infarction.(Linz et al., 1995; Gibbons, 1997).

1.4.3. The kidney

Inside the kidney, BK affects both hemodynamic and ion-exchange parameters. The vasodilatory effects of BK on the glomerule and on the medulla increase the blood flow while the contraction it elicits on mesangial cells prevents the glomerular filtration rate from rising (Bascands et al., 1994). BK also acts as a diuretic and natriuretic substance by inhibiting the increase in cAMP production elicited by the anti-diuretic hormone (ADH) inside the collector tubules (Dixon et al., 1989). Lastly, BK stimulates the secretion of renin from the kidney (Beierwaltes and Carretero, 1985).

1.4.4. The nervous system

Of the known autacoids, kinins are the most potent in producing pain. The activation of B_1 or B_2 kinin receptors inside the peripheral nervous system triggers nociceptive signals that reach the central nervous system through the sensory Aδ and C afferent fibers. Upon stimulation, the endings of these sensory fibers also release locally, inflammatory neuropeptides such as substance P and CGRP. Kinin receptors are present inside several regions of the central nervous system, mostly inside vascular beds. This vascular presence and the absence of kinins inside nerve endings or synapses, suggest that kinins

exert their effects on the central nervous system mainly through second mediators such as NO and eicosanoids. An intracerebral injection of kinins elicits numerous responses, including excitement followed by catatonia and an increase in systemic blood pressure and heart rate. The latter response involves a cardiac stimulation by the nervous sympathetic system, an increased sympathetic tone to the blood vessels and the release of serotonin and norepinephrine.(Bhoola et al., 1992)

1.4.5. Other sites of action

Other effects of BK include contraction of the iris, the uterus and the smooth muscle cells lining the bowel, increased secretion of epithelia inside the lungs, bowel and sweat glands and an increase in collagen production by fibroblasts (Bascands and Girolami, 1996).

2. Pathophysiology of myocardial infarction and of the subsequent development of heart failure

2.1. Myocardial infarction

Almost all myocardial infarctions result from coronary thrombosis occurring at the site of rupture of an atherosclerotic plaque lining the coronary wall. These atherosclerotic plaques are composed of a lipid core and a fibrous cap that protects the core from contact with the blood. Thin fibrous caps, wherein the synthesis of collagen is inhibited or its degradation enhanced, are more likely to rupture under mechanical stress and expose underlying substances that promote platelet activation and thrombin generation. The ensuing thrombus and the vasoconstriction elicited by the activated platelets may severely narrow the artery lumen and considerably reduce the perfusion of the myocardium subtended by the occluded coronary. Hypoxia, accumulation of metabolites and formation of free radicals incur damages to the cells inside the zone of myocardium, which is improperly supplied by the affected vessel. Contractions of the cardiomyocytes in the ischemic region become dyssynchronized and shorter and they ultimately cease in favor of paradoxical systolic expansions. If proper perfusion is not restored within 20 to 120 minutes and remains below the critical threshold required for cell survival, the myocytes sustain irreversible injuries and ultimately become necrotic. Necrotic myocytes entirely lose their ability to contract and therefore cease performing any work.

Slowly accruing atherosclerotic plaques usually do not lead to infarction, although they are thick and may progress toward complete occlusion of the lumina. This is probably because they allow time for collateral vessels to develop and compensate for the reduced perfusion by the main vessel. It is the sudden and complete occlusion of a coronary vessel by a thrombus superimposed on a ruptured small atherosclerotic plaque that usually results in large transmural infarctions. (Antman and Braunwald, 1997).

2.2. Heart failure: remodeling of the heart

If the infarct is of sufficient size, the overall pumping function of the ventricle is depressed and stroke volume and cardiac output fall, leading to heart failure. The infarcted heart, with its depressed stroke volume and cardiac output, is no longer able to pump blood at a rate commensurate with the requirements of the metabolizing tissues. A number of adaptive reactions will occur that aim at restoring a sufficient cardiac output, often at the expense of a long-term deterioration of the heart itself. These adaptive reactions include: (1) a remodeling of the heart; (2) an increase in afterload, blood pressure and sodium-water retention and (3) the redistribution of the subnormal cardiac output away from the skin, skeletal muscle, and kidneys with maintenance of blood flow to vital organs such as the brain and the heart itself. While these reactions are useful over the short term in supporting the heart and restoring a sufficient cardiac output, they are maladaptive over the long term. They lead to further myocardial dysfunction and thus only succeed in delaying the development of heart failure following myocardial infarction.

The process of myocardial remodeling following infarction involves an increase in myocardial mass associated with hypertrophy of the surviving myocytes, scarring of the infarcted area, changes in the quantity and quality of the extracellular matrix and the death of non-infarcted myocytes by apoptosis. Alterations in gene expression underlie most of these remodeling events.

2.2.1. Myocyte hypertrophy and apoptosis

In the infarcted heart, faced with a greater mechanical burden and various other stimuli, the non-infarcted myocytes hypertrophy. They do so by reversing to a fetal state of accelerated protein synthesis, in which they grow in both thickness and length by laying down myofibrils in parallel and sarcomeres in series. However, because the cell cycle remains blocked and no cell division occurs, this unnatural growth response of the myocytes may ultimately lead to functional impairement and cell death. In fact, later

during the hyperthrophic response, myofibrils undergo lysis, lysosomes increase in number (presumably to digest worn-out cell constituents) and the sarcoplasmic reticulum becomes distorted. Moreover, during remodeling, an increasing number of myocytes die spontaneously, in the absence of inflammation. This phenomenon, called apoptosis, also appears to be part of a fetal gene program, which becomes reactivated in the failing heart. While both hypertrophy and apoptosis are part of fetal gene programs, it is yet not clear if the two are linked together, as some stimuli can induce either of them without the other. Apoptotic cells are easily identified by the presence of double-stranded DNA breaks, which present as nucleosome-sized fragments. The study of myocardial tissue from failing hearts showed that less than 0.2% of cells undergo apoptosis at a given time. Apoptosis however is a transient event lasting perhaps only a few hours, so that the death of even 0.2% of cells per day could lead, over a period of months or years, to the loss of a large fraction of the myocyte pool. (Colucci and Braunwald, 1997; Colucci, 1997).

The enlargement of the heart induced by hemodynamic overload is an adaptive response, which allows the individual to survive. It may occur not only in pathological settings, such as essential hypertension or post-infarction, but also in the healthy individual as a response to prolonged endurance exertion. The course of cardiac hypertrophy may be divided in three stages, according to Seyle's phases of the general stress syndrome. The first stage is that of **developing hypertrophy** (Seyle's alarm reaction), where workload exceeds the normal work output of the heart. The second stage is that of **compensatory hypertrophy** (Seyle's state of resistance), a period when the work-induced growth of the heart compensates for the increased workload. The third stage is that of **uncompensated hypertrophy**, also known as heart failure (Seyle's state of exhaustion), a period when the decreasing force-generating ability of the heart no longer meets the increasing workload. (Zak, 1984)

2.2.2. Fibrosis of the extra-cellular matrix

The extra-cellular matrix of the heart also undergoes transformation during remodeling, as many factors present in the failing myocardium induce fibroblast proliferation and an

abnormal synthesis and degradation of key extracellular proteins, such as collagen. The resulting fibrosis of the failing heart, whereby myocytes are progressively replaced by connective tissue, affects the mechanical properties of the heart (e.g. impairs relaxation), restricts the delivery of nutrients to the myocytes and alters the integrin-mediated interactions between cells that are involved in biological events such as cell growth, apoptosis and gene expression. (Colucci and Braunwald, 1997; Colucci, 1997).

2.2.3. Changes at the molecular and cellular levels

Several changes at the cellular and molecular levels have been identified in the surviving – albeit hypertrophied – myocytes, which may well play a part, alongside apoptosis and fibrosis, in the long-term reduction of contractility undergone by the heart during pathological hypertrophy. Ca^{++} appears to be handled more slowly in hypertrophied myocytes. This contributes to a slower activation and relaxation of the contractile apparatus that adversely affects contractility. Alterations in the expression of enzymes and channels regulating the intracellular Ca^{++} concentration would explain this impaired handling. Changes in the contractile apparatus itself are another possible cause of the decreased contractility. A reduction of the quantity of myofibrils, as well as changes in the enzymes and proteins regulating the interaction between actin and myosin, have been noticed inside end-stage hypertrophied cells. Moreover, as late hypertrophy progresses toward heart failure, it was observed that myocardial energy reserves, as reflected by creatinine kinase activity and phosphocreatinine concentrations, were low. These lower energy reserves are suspected of further decreasing myocardial contractility at the onset of heart failure. (Colucci and Braunwald, 1997; Colucci, 1997).

2.2.4. Evolution of the scar

Another component of the remodeling undergone by the heart following infarction is the expansion of the infarcted region without additional myocardial necrosis. This increase in the size of the scar, which yields under ventricular pressure, appears to be caused by a combination of (1) slippage between muscle bundles, reducing the number of myocytes

across the infarct wall; (2) disruption of normal myocardial cells adjacent to the scar; and (3) tissue loss and tearing within the necrotic zone. Eight hours following infarction, edema and infiltration of neutrophilic polymorphonuclear leukocytes and red blood cells become evident inside the ischemic myocardium. By 24 hours, the capillaries in the involved region dilate and polymorphonuclear leukocytes accumulate, first at the periphery and then in the center of the infarct. On about the fourth day after infarction, macrophages start removing necrotic fibers, again commencing at the periphery of the infarcted region. Later, lymphocytes, macrophages and fibroblasts infiltrate between the necrotic myocytes, which become fragmented and which ultimately dissolve by 8 days. Granulation tissue first appears at the periphery of the scar by 10 days, at which time the number of polymorphonuclear leukocytes has dwindled. The removal of necrotic myocytes and their replacement by collagen fibers continues along with ingrowth of fibroblasts and blood vessels, until the fourth to sixth week following infarction. By the sixth week after infarction, the infarcted area has usually been converted into a firm connective tissue made out of collagen and interspersed with surviving intact muscle fibers. This firm connective tissue stabilizes the infarcted area against further expansion. (Antman and Braunwald, 1997).

2.2.5. Ventricular dilation

A number of stimuli are responsible for inducing cardiac remodeling following infarction, the foremost being those of mechanical nature. The inability of the infarcted ventricle to empty properly results in an increase in the end-systolic volume and, since the input of blood remains unchanged, this translates also into an increase in the end-diastolic volume. The resulting stretching of the myocardial fibers enhances their contractility and eventually restores cardiac output by the Frank-Starling mechanism. Output is restored however at the cost of a persistent ventricular dilation, which increases the radius of curvature of the wall and reduces its thickness. According to Laplace's law, the ventricular wall stress, or resistance faced by the fibers during contraction, is proportional to the wall radius and inversely proportional to the wall thickness.

$$ventricular \cdot wall \cdot stress = \frac{pressure \times wall \cdot radius^2}{2 \times wall \cdot thickness}$$

Ventricular dilation thus results in an increase of ventricular wall stress, which along with the stretching of the myocardial fibers, is a potent stimulus for hypertrophy, apoptosis, and fibroblast proliferation. Moreover, the component of ventricular dilation resulting from scar expansion during the first six weeks following infarction is purely deleterious, because it increases wall stress without stretching viable muscle fibers and enhancing their contractility. (Colucci and Braunwald, 1997; Colucci, 1997).

2.3. Heart failure: neuro-hormonal, autocrine and paracrine changes

Numerous other stimuli are also responsible for the remodeling of the heart following infarction. Most of these stimuli are molecular components of a series of neuro-hormonal, autocrine and paracrine adjustments aiming at restoring cardiac output. In order to restore cardiac output, these molecules affect not only the heart, but other organs as well, such as the blood vessels or the kidneys. It is also noteworthy that most of these molecules may also act as mediators for other stimuli. For instance, angiotensin not only induces myocyte hypertrophy by itself, but is also released by myocytes upon stretching and thus mediates the hypertrophic response of this other stimulus. Moreover, most of these stimuli operate in concert and are present in the hypertrophied or failing heart at the same time. It is therefore difficult to attribute *in vivo* responses such as, hypertrophy, apoptosis or fetal gene expression to one molecule or another, as easily as it is done *in vitro*.

2.3.1. The adrenergic system

One of the most important neuro-hormonal adjustments is the increase in adrenergic activity of the sympathetic nervous system, which helps maintain ventricular contractility through direct stimulation of the heart and through an increase in afterload. The adrenergic neurohormone norepinephrine (NE) exhibits potent vasocontrictor, chronotropic and inotropic effects. *In vitro*, NE also has the ability to cause hypertrophy, fetal gene expression and apoptosis of myocytes and activate fibroblast growth and

protein synthesis. Patients with heart failure have markedly elevated levels of NE at rest and experience greater elevations of NE during exercise, than do normal subjects. Their higher levels of NE reflect an increased activity of the adrenergic nervous system and a reduced uptake of NE by nerve endings. This augmented adrenergic outflow from the central nervous system is due, in part, to abnormal baroreflex control. In fact, with continued stimulation, as occurs in heart failure, the atrial and arterial stretch receptors become desensitized and fail to inhibit the central sympathetic nervous system properly. Over the long term, the heart itself becomes somewhat desensitized to chronic adrenergic activation, because of β-receptor downregulation and uncoupling. Moreover, the response of the heart to activation of the sympathetic nervous system becomes blunted, as cardiac NE stores are depleted and the G_i to G_s ratio of the β-receptor G-coupling proteins increases. The failing heart thus loses an important compensatory mechanism – the adrenergic response – useful in instances such as exercise. The kidney and the gut, on the other hand, do not become desensitized to adrenergic stimuli and respond to the higher levels of catecholamines released during heart failure and exercise with exaggerated vasoconstriction. Moreover, the desensitized stretch receptors mentioned earlier fail to inhibit the release of ADH and the renal efferent sympathetic nerve activity, and are thus responsible for an increase in water retention and a decrease in glomerular filtration rate. While helping to restore cardiac output at first, through an increase in afterload (vasoconstriction + water retention), and by directly stimulating (chronotropy + inotropy) and remodeling (hypertrophy) the heart, the adrenergic response places a greater stress on the heart over the long term. In addition, chronic adrenergic activation may also precipitate arrhythmias and exert a direct receptor-mediated toxic effect on the failing heart, thus favoring the vicious circle of myocyte dropout and dysfunctional hypertrophy. (Colucci and Braunwald, 1997; Colucci, 1997).

2.3.2. The renin-angiotensin system

When cardiac output declines, as it does following myocardial infarction, another neuro-hormonal response, the renin-angiotensin-aldosterone system (RAS) is activated to restore it. Figure 2 (page 35) illustrates the components of this system. Briefly,

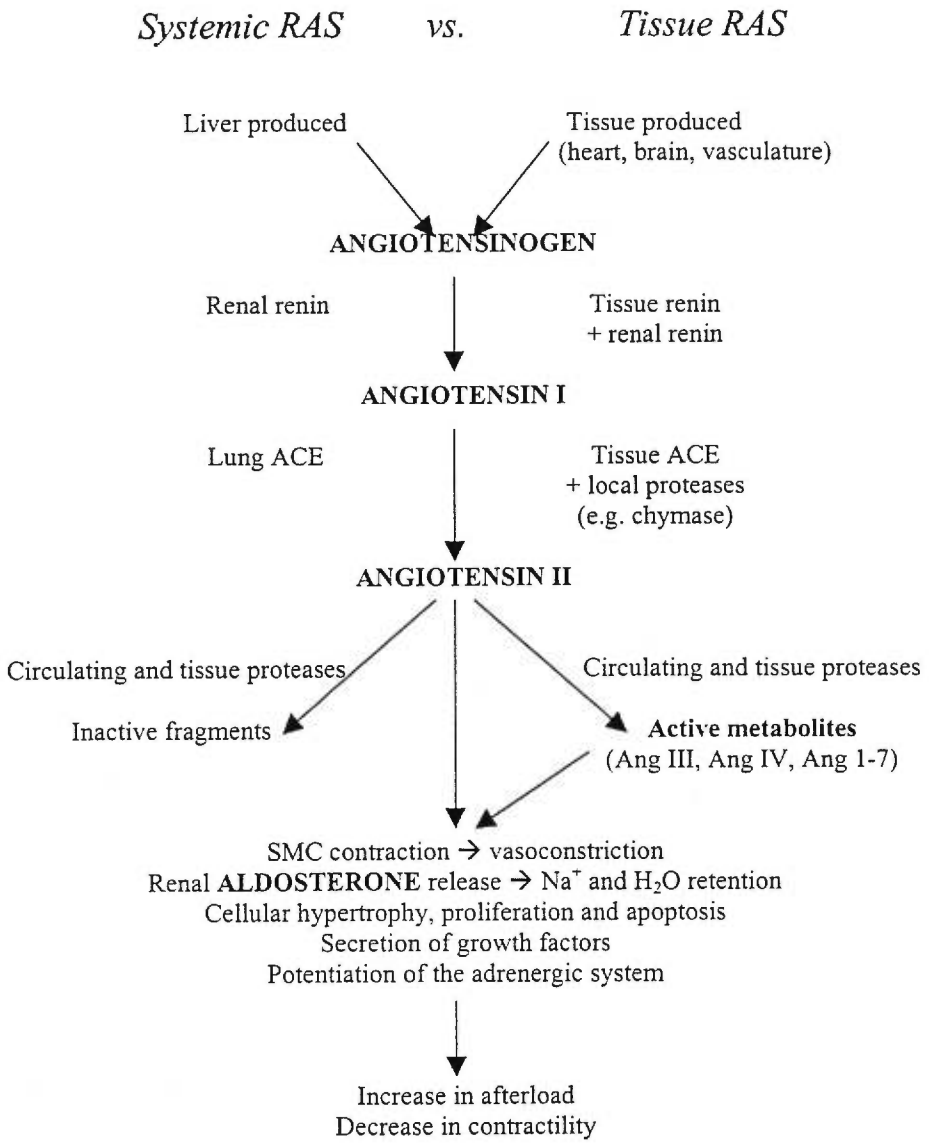

Figure 4: The systemic and tissue components of the renin angiotensin system (RAS). The systemic components are illustrated on the left-hand side, while the tissue components are shown on the right-hand side. Adapted from Colucci and Braunwald, 1997.

angiotensinogen is converted by renin to angiotensin I, which is further converted by ACE into angiotensin II, whose many effects include stimulating the production of aldosterone. Angiotensin II and aldosterone are the main active substances of the RAS, the former contributing to excess vasoconstriction and the latter to the retention of salt and water. The adrenergic nervous system and the RAS operate in concert following myocardial infarction. In fact, both systems are activated by vascular baroreceptors

responding to a decline in cardiac output. The stimulation of the β-adrenergic receptors in the kidney contributes to the release of renin in the circulation, and angiotensin II enhances the release of NE by the sympathetic nervous system. All of the necessary components of the RAS are present in several organs and tissues, including the vasculature, heart and kidneys. When compared to the circulating RAS, tissue RAS contains other proteases besides ACE that are able to convert angiotensin I to angiotensin II. Moreover, the local renin-angiotensin system appears to be upregulated inside tissues during cardiovascular disease. The expression of ACE, for instance, is increased within the heart in the context of ventricular remodeling and heart failure post-myocardial infarction. In addition to its potent vasoconstrictor activity, angiotensin II also promotes cell growth and alters gene expression in a variety of cells. Inside the heart, angiotensin II causes apoptosis, increases protein synthesis in myocytes and DNA synthesis in fibroblasts, by reversing these cells to fetal gene programs and by stimulating the secretion of various growth factors. As mentioned earlier, angiotensin is also released by the cardiac myocytes upon stretching and is thus in part responsible for mediating the hypertrophic, myeloproliferative and apoptotic effects of stretching on the surrounding cells. Angiotensin II and its active metabolites Ang 1-7, Ang III and IV act on two receptor subtypes AT_1 and AT_2. Inside the heart, the AT_1 receptors appear to mediate the apoptotic, hypertrophic and proliferative effects of angiotensin II, as these effects can be blocked by a selective antagonist for this receptor. In end-stage heart failure, when both circulating and tissue RAS are activated, angiotensin receptors become downregulated. (Colucci and Braunwald, 1997; Colucci, 1997).

2.3.3. The anti-diuretic hormone

Arginine vasopressin (AVP) – also called anti-diuretic hormone (ADH) – is a pituitary hormone that regulates plasma osmolality by reducing free water clearance. It exerts its effects by acting on two receptor subtypes V_1 and V_2. Selective inhibition of these receptors suggests that ADH regulates free water clearance through the V_2 receptor and, in addition, exerts a vasoconstrictor effect through the V_1 receptor. In heart failure following myocardial infarction ADH levels are abnormally elevated and fail to decline

with reductions in blood osmolality. Like NE and angiotensin, ADH is thus responsible for an increase in preload and afterload, which delays the development of heart failure by restoring cardiac output and perfusion pressure over the short term and increasing the burden on the heart over the long term. (Colucci and Braunwald, 1997).

2.3.4. The natriuretic peptides

Three natriuretic peptides have been identified in humans, atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP) and C-natriuretic peptide (CNP). ANP and BNP have natriuretic and vasodilatatory effects, which counteract the water-retaining and vasoconstrictor effects of the adrenergic, renin-angiotensin and ADH systems. Both ANP and BNP are stored inside the myocardium, the former mainly in the right atrium and the latter mainly in the ventricles. They are produced and are released into the bloodstream in response to an increase in wall tension. CNP is located mainly in the vasculature and its physiological role is not yet clear. During heart failure, plasma levels of ANP and BNP increase significantly. This spillover is the result of an increase in the ventricular and atrial expression of both ANP and BNP. The myocardial content of CNP also becomes significant. Three types of receptors for natriuretic peptides have been identified: A, B and C. The A and B receptors mediate the vasodilatatory and natriuretic effects of the peptides, while the C receptor plays a role in regulating the available levels of the peptides along with their catabolizing enzyme, the neutral endopeptidase. In addition to vasodilation and natriuresis, the natriuretic peptides also directly inhibit myocyte and smooth muscle hypertrophy and interstitial fibrosis. Contrary to the adrenergic, RAS and ADH systems, natriuretic peptides do not merely delay the onset of heart failure, but steer the heart away from it - this, of course, at the expense of reducing the compensatory response that follows myocardial infarction. (Colucci and Braunwald, 1997; Colucci, 1997).

2.3.5. Endothelin

Angiotensin, NE and cytokines stimulate the release of endothelin from endothelial cells and locally inside tissues. Endothelin is a potent and long-lasting vasoconstrictor substance, which also exerts important effects on myocyte growth and reversal to fetal phenotypes and alters the synthesis and degradation of collagen in the myocardium. Three endothelin peptide isoforms, endothelin-1, -2 and -3, and two subtypes of endothelin receptors, ET_A and ET_B, have been identified so far. While there is evidence that endothelin-2 may possibly function as a mediator in the kidney and that endothelin-3 may act as a mediator in the gut and nervous system, endothelin-1 is the major isoform generated in blood vessels and appears to be of greatest importance in cardiovascular regulation. The ET_A receptor predominates on smooth vascular muscle cells and is responsible for causing vasoconstriction. It is also the major receptor subtype in the heart. Plasma endothelin levels increase following myocardial infarction and correlate with the severity of damage. However, endothelin acts as a paracrine and autocrine mediator and its secretion by endothelial cells, for instance, is largely abluminal (i.e. toward the adjacent vascular smooth muscle). Moreover, like angiotensin, endothelin can be produced by a variety of cells inside the heart and both endothelin-1 and its receptors are upregulated inside the remodeling myocardium. Endothelin thus plays a role, along with the adrenergic and renin-angiotensin systems, in increasing afterload, remodeling the heart and delaying the onset of heart failure following myocardial infarction. (Colucci and Braunwald, 1997; Colucci, 1997).

2.3.6. Growth factors and cytokines

Several other peptide mediators, including peptide growth factors and inflammatory cytokines, appear to play an important role in mediating the changes undergone by the heart and vasculature following myocardial infarction and during heart failure. Peptide growth factors, such as tumor growth factor β, are expressed by many cell types, including cardiac myocytes and fibroblasts. Their expression increases in response to mechanical stress (ex. stretching) as well as substances such as NE. Peptide growth factors have been shown to cause hypertrophy and cell proliferation associated with induction of fetal genes. Inflammatory cytokines such as TNFα, IL-1α, IL-1β, IL-2 or

IL-6 also exert long-term effects on the remodeling of the myocardium and vasculature. While some studies on human and animal models demonstrated a cardiodepressive effect of pro-inflammatory cytokines on the failing heart, serum concentrations of the cytokines did not always correlate with the presence or degree of clinical heart failure. Several cell types inside the myocardium, including myocytes and fibroblasts have receptors for and respond directly to inflammatory cytokines. IL-1β for instance can directly induce hypertrophy in cultured cardiac myocytes. Cytokines also appear to play an important role in the regulation of the NO pathway, a likely secondary mechanism by which they affect the heart. Moreover, their important role in immune reactions also suggests that an immunological mechanism, such as autoimmunity, may be involved in the pathogenesis of heart failure. At the present time however, the role of inflammatory cytokines in mediating heart failure remains to be determined. (Colucci and Braunwald, 1997; Colucci, 1997; Blum and Miller, 1998).

2.3.7. Nitric oxide

Nitric oxide (NO) can affect the myocardium in several ways. *In vitro*, NO inhibits the contractile response to β-adrenergic stimulation and being a free radical induces apoptosis in cardiac myocytes. NO is produced in several cell types, by nitric oxide synthase, of which two isoforms, one constitutive (cNOS) and one inducible (iNOS), are present in the human myocardium. Inflammatory cytokines are potent inducers of NO production via induction of iNOS. NO is metabolized by other free radicals, so that its rate of catabolism depends on the redox state of the tissue. An increase in oxidative stress will mitigate the bioactivity of NO. During hypertrophy and at the onset of heart failure, oxidative stress and levels of oxygen free radicals appear to be increased in the myocardium, most likely under the influence of mechanical stress (stretching) and the effects of inflammatory cytokines (TNFα). Such increases in oxydative stress and oxygen free radicals are associated with the induction of apoptosis, increased protein synthesis and reversal to fetal gene expression in both cardiac myocytes and fibroblasts. (Colucci, 1997; Gibbons, 1997).

3. ACE Inhibitors

The various pharmacologic agents used to delay the onset of heart failure after myocardial infarction aim primarily at reducing afterload and impeding the remodeling of the heart, as both these responses of the cardiovascular system have proven to be maladaptive, when clinically activated. ACE inhibitors (ACEi), by altering the balance between the vasoconstrictive and salt-retentive properties of Ang II and the vasodilatatory and natriuretic propertries of BK, are potent anti-hypertensive agents effective in reducing afterload, pre-load and ventricular wall stress. ACEi also exert a vagomimetic effect which could explain why tachycardia is absent despite peripheral vasodilation. This may contribute to their anti-arrhythmic properties.

3.1. ACE inhibitors in congestive heart failure

ACEi are able to slow or reverse the remodeling of the heart, not only indirectly by reducing afterload, but also directly by limiting the formation of Ang II, which, as we have seen, promotes hypertrophy, apoptosis and fibrosis in the heart. In fact, the beneficial effects of ACEi on heart remodeling have been demonstrated to occur with doses below those required for a hypotensive effect. Moreover, limiting the formation of Ang II also lowers the activity of other maladaptive neuro-humoral responses, as Ang II enhances the release of NE, vasopressin and endothelin. Several large clinical trials have shown ACEi to reduce cardiovascular mortality after myocardial infarction and to slow the progression of heart failure. ACEi have also been shown to be the most effective agents in reversing ventricular hypertrophy in patients with hypertension. (Parmley, 1998; Kelly and Smith, 1997; Opie and al., 1995).

3.2. ACE Inhibitors as anti-hypertensive agents

ACEi have been proven beneficial in the treatment or prevention of numerous cardiovascular events, other than congestive heart failure. In hypertensive subjects, ACEi

effectively lower systolic and diastolic blood pressures. While a higher pre-treatment plasma renin activity (PRA) favors a greater initial reduction in blood pressure, with long-term therapy the anti-hypertensive effect of ACEi no longer correlates with PRA. This suggests that the long-term hypotensive effect of ACEi may rely more on the tissue arm of the RAS, the kallikrein-kinin system or the production of vasodilatatory prostaglandins. Blacks appear to be less responsive than whites to the antihypertensive effect of ACEi. This racial difference could be explained by a higher baseline ACE activity in blacks requiring higher doses of ACEi and by a greater incidence of high PRA in whites. (Brown and Vaughan, 1998).

3.3. ACE Inhibitors as vasoprotective agents

Another clinical indication for ACEi is preventing atherosclerotic vascular disease. To date ACEi have been shown to decrease the frequency of ischemic events only in patients with left ventricular dysfunction or congestive heart failure, however experimental evidence suggests that they may retard atherosclerosis and prevent ischemic events in patients with normal ventricular function as well. In fact, ACEi have been shown to reverse endothelial vasomotor dysfunction in normotensive patients with non-insulin-dependent diabetes mellitus, to reduce the extent of vascular lesions in various animal models of atherosclerosis and to promote ischemic pre-conditioning. ACEi likely improve endothelial function by influencing the activity of three vasoactive systems: Ang II, BK and NO. They reduce the vasoconstrictive, myoproliferative and superoxide radical generating effects of Ang II while simultaneously enhancing the vasodilatatory and vasoprotective effects of BK and NO (discussed earlier) – ACEi improve NO bioactivity by enhancing its BK mediated induction and by reducing its oxidative catabolism. By altering the balance between Ang II and BK, ACEi also enhance the secretion and activity of the plasminogen activator, a fibrinolytic agent which may help prevent ischemic events. (Brown and Vaughan, 1998; Parmley, 1998; Gibbons, 1997).

3.4. ACE Inhibitors as reno-protective agents

ACEi have also been shown to reduce the loss of kidney function occurring in a variety of renal diseases including diabetic nephropathy, glomerulonephritis, interstitial nephritis and nephrosclerosis. This protective effect of ACEi, which is independent of the severity of renal insuficiency, may be explained by a reduction in glomerular pressure, resulting from a decrease in arterial pressure and the selective dilation of the efferent arterioles, and by the reduction of the trophic effects of Ang II on mesangial cell growth and matrix production. (Brown and Vaughan, 1998).

3.5. Adverse effects

Adverse effects of ACEi include hypotension, hyperkalemia, reversible renal hypoperfusion, cough and angioedema. ACEi may be overly hypotensive in patients in whom hypertension results mostly from excessive renin secretion, such as occurs in volume depletion, low sodium intake or diuretic use. ACEi may also cause hyperkalemia because of a decrease in aldosterone. This effect is significant however only in patients with impaired renal function, or who are taking potassium sparing diuretics or potassium supplements. In patients with impaired renal function, in which glomerular filtration depends to a great extent on efferent arteriole tonus, ACEi may severely reduce glomerular filtration and precipitate renal failure by altering the balance between Ang II and BK and thus dilating the efferent arterioles. Cough is another side effect of ACEi, whose mechanism is unknown, but appears to involve increased levels of BK, substance P and stimulation of the vagal C fibers. Cough is more frequent among women than men, and more frequent among asians (≈44%) than whites (≈10%). A rare, but potentially life-threatening effect of ACEi is angioedema, the localized swelling of the mouth, lips, throat, nose or other parts of the face. It occurs in 1 to 2 patients per 1000 in the white population and appears to be more frequent in blacks. This adverse effect likely involves the accumulation of des-Arg^9-BK resulting from a decrease in aminopeptidase P activity, which appears to be genetic, as it afflicts members of the same family. This accumulation

of des-Arg^9-BK could also be responsible for two other acute side effects associated with ACEi, namely hypotensive reactions in ACEi-treated patients undergoing hemodialysis with negatively charged membranes, and anaphylactoid reactions in ACEi-treated patients during blood transfusions. (Brown and Vaughan, 1998; Blais et al., 1999 a and b; Cyr et al., 1999).

3.6. Role of endogenous BK in the effects of ACE Inhibitors

The effects of ACEi may be mediated by several mechanisms. To date, only the inhibition of the ACE-performed conversion of Ang I to Ang II has clearly been proven to play a long-term role in humans. Other potential mechanisms of ACEi include inhibition of BK catabolism and the promotion of alternative metabolic pathways for Ang I and BK, which result in alternative degradation products such as the vasodilator Ang 1-7 or the long-lasting B_1 agonist des-Arg^9-BK. Moreover these mechanisms may work in concert, as for instance, BK mediates part of the vasodilatatory effects of Ang 1-7 (Brosnihan and al., 1996). The contribution of BK to the effects of ACEi in humans is uncertain and has been the subject of several studies. The return of PRA and Ang II plasma levels to pre-treatment values during long-term ACE inhibition and the increase in effectiveness of ACEi (e.g. hypotensive effect) with long-term administration, suggest that BK and the tissue arms of the kallikrein-kinin and renin-angiotensin systems contribute to the actions of ACEi. Another indicator supporting a role for endogenous BK is that with long-term treatment ACEi may inhibit other BK-degrading enzymes besides ACE, such as NEP in the rat kidney or aminopeptidase P in the pig kidney (Drummer, 1990; Hooper, 1992). Accurate measurements of endogenous BK levels are however technically baffling, because the sampled blood or tissues contain all the necessary components to generate and destroy this short-lived peptide. Thus, endogenous BK concentrations have been reported to be either increased or unchanged during ACE inhibition. The use of exogenous BK circumvented this assay problem and exogenous BK was shown to be potentiated by ACE inhibition in several human and animal models. In fact, ACEi potentiate the actions of ACE-resistant BK analogs as well, indicating that some kininase-independent mechanisms of action are also involved, such as an allosteric

facilitation of kinin-receptor activation or a buffering effect on an ACE-like binding site of BK. These observations with exogenous BK or its analogs however do not indicate whether endogenous BK plays a part in the actions of ACEi. Other strategies allow to asses, albeit indirectly, the respective roles of endogenous BK and Ang II in the effect of ACEi. They include the addition of selective BK antagonists or Ang II to ACE inhibition, the comparison of ACEi with selective Ang II antagonists and the use of animal models deficient in kinins or B_2 receptors. (Brown and Vaughan, 1998; Linz et al., 1995).

3.6.1. Vasoprotective effect

Endogenous BK likely participates in the vasoprotective effects of ACEi, as B_2 antagonism reduced the anti-proliferative and anti-hypertrophic effect of these agents on blood vessels following balloon injury to levels similar to simple AT_1 antagonism (Farhy et al., 1989 and 1993).

3.6.2. Cardioprotective effect

Endogenous BK likely contributes to the cardioprotective effects of ACEi in the setting of myocardial infarction, as revealed by the following *in vitro* and *in vivo* animal studies. In isolated rat hearts, perfusion of ACEi and BK have similar cardioprotective effects during ischemia and reperfusion (Linz et al., 1995). Moreover, ACE inhibition enhances the local kinin levels in the cardiac muscle (Campbell et al., 1993). B_2 antagonism abolishes the anti-arrhythmic and metabolic effects of ACEi following infarction in the isolated rat heart and the ACEi-confered delay in the development of myocardial infarction in the dog *in vivo* (Linz et al., 1990; Martorana et al., 1990). Moreover, B_2 antagonism reverses the anti-growth effect of ACEi after non-transmural necrosis in the dog myocardium and attenuates the anti-arrhytmic effect of ischemic pre-conditioning in the rat heart (McDonald et al., 1995; Vegh et al., 1994). Furthermore, addition of Ang II during ACE inhibition does not significantly reverse the attenuation of reperfusion arrythmias by inhibition of ACE in the ischemic zone (Shimada et al., 1996).

3.6.3. Hypotensive effect

Endogenous BK also contributes to the hypotensive effect of ACEi. In kininogen-deficient rats ACEi are less potent hypotensors than in kininogen-replete rats and moreover, co-administration of a B_2 antagonist has no effect on the former model while it does attenuate the anti-hypertensive effect in the latter (Danckwardt et al., 1990). However, this contribution of BK seems to be effective only in high renin-models of hypertension, where ACEi are most potent. In rats with renovascular hypertension – a model of high-renin hypertension – B_2 antagonism resulted in a 20-30% decrease of the anti-hypertensive effect of ACEi (Bönner, 1997). On the other hand, in spontaneously hypertensive rats – a model with normal to low renin levels – B_2 antagonism does not attenuate the anti-hypertensive effect of ACEi. (Aubert et al., 1987; Waeber et al., 1986).

3.6.4. Anti-hypertrophic effect

The participation of endogenous BK to the anti-hypertrophic effect of ACEi is also affected by the renin status of the model. This is of particular interest when considering that this status may change during ACEi treatment, as plasma renin activity decreases with time. In rats with renovascular hypertension, low sub-hypotensive doses of ACEi reduce vascular and cardiac hypertrophy and this effect is abolished by B_2 antagonism. However, B_2 antagonism does not abolish the antihypertrophic effect of higher hypotensive doses of ACEi (Linz et al., 1992). This indicates that although BK contributes to the anti-hypertrophic effect of ACEi in high-renin hypertension, this contribution is minor when compared to that of the reduction of blood pressure. In rats with pressure overload induced by aortic banding – another high-renin model – B_2 antagonism attenuates the effects of ACEi in preventing the development of left ventricular hypertrophy, but does not attenuate the effect of ACEi in reversing established hypertrophy. Furthermore, chronic administration of a low non-hypotensive dose of BK prevents the development of left ventricular hypertrophy, but does not reverse established hypertrophy in rats with pressure overload (Linz et al., 1995). The contribution of BK to the anti-hypertrophic effect of ACEi in **high-renin hypertension** is

therefore relevant only during the prevention phase and is later outweighed by other mechanisms, such as the attenuation of Ang II, during the regression period.

In spontaneously hypertensive rats, hypotensive doses of ACEi prevent the development of hypertension and left ventricular hypertrophy, but B_2 antagonism did not reverse this effect. Low, sub-hypotensive doses of ACEi had no such preventive effect in spontaneously hypertensive rats although they reduced already established left ventricular hypertrophy. On the other hand, in spontaneously hypertensive stroke-prone rats – another normal to low renin model – cardiac function and metabolism were improved by both high and low-dose ACEi treatments and B_2 antagonism abolished this effect (Gohlke et al., 1994). Hence, in **normal to low -renin hypertension**, BK might well contribute to the anti-hypertrophic effect of ACEi. This contribution however is minor and irrelevant during the prevention phase and remains to be determined in the regression phase.

4. ACE-NEP Inhibitors

4.1. NEP inhibitors

As we have seen, NEP is a ubiquitous membrane-bound peptidase whose many substrates include enkephalins, ANP and BK. Its important role in the degradation of enkephalins inside the central nervous system and in the periphery, makes the enkephalin-potentiating NEP inhibitors (NEPi), promising analgesic, antidepressive and antidiarrheic agents (Roques et al., 1995). Similarly, the role of NEP in the degradation of ANP and BK points toward a potential diuretic, hypotensive and cardioprotective effect of NEPi. Renal and vascular NEP activity, along with clearance through C-receptors, were shown to be the most important catabolic pathways of the natriuretic peptides (Ruskoaho, 1992). Inhibition of NEP increases circulating ANP levels and potentiates the natriuretic, diuretic, anti-hypertrophic and blood pressure-lowering effects of natriuretic peptides, without changing potassium elimination (Roques et al., 1995). Theoretically, BK should also play a role in the renal and blood pressure-lowering effects of NEPi, as it is another hypotensive, diuretic and natriuretic peptide most likely potentiated by NEP inhibition. The actual role of endogenous BK in NEP inhibition however remains unclear and requires further studies.

The hypotensive, natriuretic and diuretic effects of NEPi were not reduced by B_2 antagonism in a low-renin model of hypertension (DOCA-salt rats) and the effects of NEPi in this model were similar to those in a kininogen-kinin-deficient model (Pham et al., 1996 and 1992). Moreover, B_2 antagonism did not alter the acute anti-hypertensive effect of NEPi in hypertensive hamsters with compensated heart failure (Trippodo et al., 1995a). These results suggest that NEPi do not depend on BK for their acute effects. This could be explained by the fact that ACE would readily compensate for the reduced degradation of BK during NEP inhibition. BK could nevertheless play a role in long-term NEPi treatment, as chronic B_2 antagonism increases blood pressure in DOCA-salt rats

and reduces the anti-hypertensive effect of ACEi in rats with renovascular hypertension (Pham et al., 1996).

4.2. Simulataneous inhibition of NEP and ACE

NEPi when given alone are poor anti-hypertensive drugs, probably because they also potentiate two vasoconstrictor substrates of NEP, endothelin-1 and Ang II, and because ACE readily compensates for their reduction in BK degradation (Ferro and al., 1998; O'Connell et al., 1993; Richards et al., 1992). However, when NEPi are given together with ACEi, they induce greater reductions in blood pressure than ACEi alone (Pham et al., 1993). This synergistic effect of dual ACE and NEP inhibition on blood pressure is due to the complementary physiological roles of angiotensin attenuation and ANP potentiation, as well as to an increase of the ANP-elicited inhibition of renin release (Ruskoaho, 1992) and to the potentiation of BK, whose two main catabolic pathways are blocked and prevented from compensating for one another. Moreover, whereas ACEi alone lower blood pressure more effectively in renin-dependent forms of hypertension, NEPi do so in salt- and volume-dependent forms of hypertension, where renin activity is usually low (Pham et al., 1993). Coinhibition of ACE and NEP should thus lower blood pressure in a broader range of conditions than inhibition of either ACE or NEP, independent of the activity of the RAS or of the degree of salt retention.

4.3. Dual vasopeptidase inhibitors

In order to take advantage of the potentiating effects of NEPi on ACEi, via the ANP and BK pathways, dual vasopeptidase inhibitors, which are single molecules able to inhibit both ACE and NEP, were developed. These compounds were rationally designed through structure-activity studies, by taking into account the structural characteristics of the active sites of both enzymes. As expected, dual NEP/ACE inhibitors elicit synergistic cardiovascular effects that mimic those observed in response to the co-administration of selective ACE and NEP inhibitors (Trippodo et al., 1995b). They inhibit both ACE and

NEP *in vitro* with nanomolar potencies and abolish the pressor response to Ang I *in vivo* with similar (or greater) potency and duration to those of ACEi. Dual ACE/NEP inhibitors lower blood pressure, increase ANP and BK levels and potentiate the hypotensive and natriuretic responses to ANP as well as the hypotensive response to BK in animal models regardless of renin or salt status (Gonzales-Vera et al., 1995). In fact, if the ACE inhibitory activity lowers blood pressure in animals with high renin, the NEP inhibitory activity does so in animals with low renin, and both activities act in concert in animal models with intermediate levels of renin (Trippodo et al., 1995b; French et al., 1995). Moreover, because ANP suppresses renin release, dual ACE/NEP inhibition elicits a lower initial increase in PRA than selective ACE inhibition (Bralet et al., 1994).

Dual NEP/ACE inhibitors have equally been proven effective in animal models of compensated heart failure. They reduce cardiac preload and afterload while augmenting cardiac output in a cardiomyopathic hamster model that showed little or no acute cardiovascular response to selective NEP or ACE inhibition alone (Trippodo et al., 1995 a and b). When compared to an ACEi, a dual ACE/NEP inhibitor was more effective in preventing the development of heart failure in rats following myocardial infarction, as measured by the attenuation of hypertrophy and the reduction in ventricular ANF levels (Bralet et al., 1994). This superior cardioprotective effect of the dual ACE/NEP inhibitor (alatriopril) occurred in spite of a lesser inhibition of angiotensin-induced hypertension and of a lesser potentiation of bradykinin-induced hypotension when compared to the ACEi (captopril). Interestingly both treatments inhibited ACE to the same extent as determined by plasma ACE activity. Thus, the difference in the cardioprotective activity between the two inhibitors is not due to their effect on plasma ACE, but rather to their diverse effects on tissue ACE and on NEP. Although both inhibitors feature nanomolar Ki for ACE, the dual NEP/ACE inhibitor was administered at a dose 10-fold higher than the simple ACE inhibitor. However, the ACE inhibitor (captopril) reduces cardiac hypertrophy in similar models of myocardial infarction at doses both higher or lower than the one used in this study, so that the dosage is unlikely to account for its inferior cardioprotective activity when compared to the dual ACE/NEP inhibitor. The superior cardioprotective effect of the dual ACE/NEP inhibitor is most likely due to its

potentiation of the hypotensive, *antihypertrophic*, diuretic and natriuretic actions of ANP as well as of the vasodilatatory, antiarrythmic and metabolic actions of BK. Moreover, this effect on the RAS is probably more important at the tissue level than in the circulation, since the dual ACE/NEP inhibitor proved less effective than the ACE inhibitor at potentiating the hypotensive effects of intra-venously administered exogenous BK. As endogenous BK remains difficult to assay, it would be interesting to study the effect of a dual ACE/NEP inhibitor on the enzymatic activities of cardiac ACE and NEP, two of the most important enzymes in the regulation of kinin levels inside the heart.

ACE and NEP being two of the most important BK-degrading enzymes, inhibiting both of them should potentiate BK more than either inhibition alone by preventing either enzyme from compensating the blocked catabolism of the other. Experimental results so far support a role for BK in the acute hemodynamic effects of dual ACE/NEP inhibitors. Whether this role is more important than in simple ACE inhibition, remains to be seen. In hypertensive hamsters with compensated heart failure, B_2 antagonism significantly blunted the decrease in preload, but did not affect the decrease in afterload, after dual ACE/NEP inhibition (Trippodo et al., 1995a). In DOCA-salt rats, dual ACE/NEP inhibition increased urinary BK concentrations and potentiated the hypotensive response to exogenous BK. It did not however potentiate the diuretic and natriuretic responses to exogenous BK, probably because of hypotension-compensating mechanisms (Pham et al., 1996).

5. Purpose of the study

ACEi have been shown to significantly decrease mortality and morbidity following myocardial infarction and to slow – and in some instances even reverse – the development of left ventricular hypertrophy. Part of these beneficial effects of ACEi have been attributed to a decrease in the metabolism of endogenous BK, whose cardiac levels increase during ACE inhibition. However, at this time, only indirect evidence exists for such a protective mechanism, and further evidence is required concerning this role of endogenous BK.

This study has a two-fold purpose. First, having recently characterized the metabolism of BK inside the normal heart, we will now define the effect of myocardial infarction and left ventricular hypertrophy on the cardiac metabolism of BK. For this purpose, we will use a rat model of myocardial infarction, which is physiologically relevant. Secondly, in the same rat model, we will also define the participation of ACE in the degradation of BK, by using an ACEi called enalaprilat. Moreover, we will also test the protective effect of omapatrilat on the cardiac metabolism of BK and compare it to that of enalaprilat. Omapatrilat is a dual vasopeptidase inhibitor, which inactivates simultaneously both ACE and NEP. This experimental approach is useful not only to objectivate the participation of BK in the cardioprotective effects of ACEi, but also to clearly establish the basis for a therapeutic use of this new class of dual vasopeptidase inhibitors.

Chapter 2 :

BRADYKININ METABOLISM IN THE POSTINFARCTED RAT HEART: ROLE OF ANGIOTENSIN-CONVERTING ENZYME AND NEUTRAL ENDOPEPTIDASE 24.11

BRADYKININ METABOLISM IN THE POSTINFARCTED RAT HEART: ROLE OF ANGIOTENSIN-CONVERTING ENZYME AND NEUTRAL ENDOPEPTIDASE 24.11

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1. Abstract

The respective role of angiotensin-converting enzyme (ACE) and neutral endopeptidase 24.11 (NEP) in the degradation of bradykinin (BK) has been studied in the infarcted and hypertrophied rat heart. Myocardial infarction (MI) was induced in rats by left descendant coronary artery ligature. Animals were sacrified and hearts were sampled respectively 1, 4 and 35 days post-MI. BK metabolism was assessed by incubating synthetic BK with heart membranes from sham hearts, and infarcted (scar) and non-infarcted regions of infarcted hearts. The half-life ($t_{½}$) of BK showed significant differences among the 3 types of tissue at 4 days [sham heart (114 ± 7 sec) > non-infarcted region (85 ± 4 sec) > infarcted region (28 ± 2 sec)] and 35 days post-MI [sham heart (143 ± 6 sec) = non-infarcted region (137 ± 9 sec) > infarcted region (55 ± 4 sec)]. No difference was observed at 1 day post-MI. The participation of ACE and NEP in the metabolism of BK was defined by preincubation of the membrane preparations with enalaprilat, an ACE inhibitor, and omapatrilat, a vasopeptidase inhibitor that acts by combined inhibition of NEP and ACE. Enalaprilat significantly prevented the rapid degradation of BK in every tissue type and at every sampling time. Moreover, omapatrilat significantly increased the $t_{½}$ of BK compared to enalaprilat in every tissue type and at every sampling time. These results demonstrate that experimental MI followed by left ventricular dysfunction significantly modifies the metabolism of exogenous BK by heart membranes. ACE and NEP participate in the degradation of BK since both enalaprilat and omapatrilat have potentiating effects on the $t_{½}$ of BK.

Keywords: bradykinin, angiotensin-converting enzyme, neutral endopeptidase, vasopeptidase inhibitor, left ventricular hypertrophy.

2. Introduction

Angiotensin-converting enzyme (ACE) inhibitors have proven to prolong the survival of postinfarction patients with left ventricular dysfunction (25). Several mechanisms have been proposed to explain this effect, one of which is the prevention of adverse ventricular remodeling postinfarction. Although the inhibition of the production of angiotensin II plays a role in this effect, there is mounting evidence that at least part of the beneficial effects of ACE inhibitors postinfarction are the result of the inhibition of bradykinin (BK) metabolism, which in turn increases nitric oxide and prostaglandin levels (18). Paradoxically, the cardiac metabolism of BK in the acute, subacute and chronic postinfarction periods has never been measured. Also, the effects of ACE inhibitors on the cardiac metabolism of BK in these settings have never been evaluated.

It has been proposed that a potentiation of the effect of BK by ACE inhibitors in the postinfarction period could be an important mechanism by which ACE inhibitors prevent scar expansion postinfarction (19). The effect of ACE inhibitors on BK metabolism in the area of the necrosis and scar appears then to be particularly important to evaluate. Although ACE inhibitors reduce cardiac collagen production in the postinfarction period (19, 37), which in turn should promote scar expansion, ACE inhibitors might also reduce scar expansion, thus contributing to their beneficial effects. The effect of ACE inhibitors on BK metabolism in the remaining viable myocardium also appears to be important as there is mounting evidence that BK plays an essential role in preventing chronic left ventricular dilatation postinfarction (19).

BK is the prototype of kinins, a family of powerful bioactive autacoids released from their precursors called kininogens (4, 27). BK exerts its pharmacological activities by activating the B_2 receptors, which are widely distributed throughout mammalian tissues (4, 13). There has recently been a renewed interest in BK owing to its cardiovascular activities consisting mainly of vasodilatory and anti-proliferative effects (18). BK is a

short-lived peptide due to its rapid metabolism by different peptidases. In vitro, many enzymes are able to metabolize BK. These enzymes are mainly metallopeptidases, but also include serine peptidases and proteases, astacin–like metallopeptidases and cathepsins (12). The nature of the enzymes involved in the metabolism of BK in vivo, and their relative importance, depends on the biological medium considered. Recently, we have shown that angiotensin-converting enzyme (ACE, peptidyl dipeptidase A, kininase II, EC 3.4.15.1) is the main enzyme responsible for the metabolism of BK, not only in rat and human serum (9), but also at the coronary vascular bed level (11) and in cardiac membrane preparations of normal hearts of different animal species (5). ACE metabolizes first BK into $BK_{[1-7]}$ and, in a second step, $BK_{[1-7]}$ is degraded into $BK_{[1-5]}$ (12). Besides ACE, other enzymes metabolize BK. Kininase I, a generic name for different plasma and cell membrane carboxypeptidases, is responsible for the metabolism of BK into its active metabolite des-Arg^9-BK (12, 32). In the serum, as well as in the heart, the kininase I pathway is a minor metabolic pathway of BK, which becomes evident only when ACE is inhibited (5, 9). Finally, we have also demonstrated that neutral endopeptidase 24.11 (NEP, neprilysin, EC 3.4.24.11) plays an important role in the degradation of BK at the endothelial level. Like the kininase I pathway, the NEP pathway becomes evident only if ACE has been previously inhibited (11). NEP metabolizes BK into $BK_{[1-7]}$ and, then, $BK_{[1-7]}$ is cleaved into $BK_{[1-4]}$ (12).

A new class of compounds, the vasopeptidase inhibitors, has recently been developed which not only inhibits the activity of ACE but also the activity of NEP (28, 35). In addition to their protective effect on natriuretic peptides (28, 31), these dual ACE/NEP inhibitors would be expected to increase BK levels more than ACE inhibitors alone. These drugs are now being investigated in clinical trials for use in hypertension and congestive heart failure, and their use in the early and late postinfarction period is being considered. Given the indirect evidence for a role of BK in the cardioprotective effects of ACE inhibitors, it would appear essential to evaluate the effects of these new ACE/NEP inhibitors on the metabolism of BK both in healthy and pathologic cardiac tissue. Omapatrilat (28) is a member of this new class of therapeutic agents (vasopeptidase

inhibitors) and, in this study, its effects on BK metabolism in healthy and postinfarction (early and late) cardiac tissue were evaluated and compared to the effects of an ACE inhibitor. The experimental model used, that of the postinfarction rat, is clinically relevant. (26).

3. Materials and Methods

3.1. Drugs, peptides and reagents

BK and des-Arg^9-BK were purchased from Peninsula Laboratories (Belmont, CA). The ACE inhibitor enalaprilat (IC_{50} = 4 nM) was obtained from the pharmacy of the Institut de Cardiologie de Montréal (Montréal, QC). The vasopeptidase inhibitor omapatrilat that acts by combined inhibition of ACE (IC_{50} = 5 nM) and NEP (IC_{50} = 9 nM) was provided for research purposes by Bristol-Myers Squibb (Princeton, NJ). Ketamine HCl was obtained from Rogar/STB (Montréal, QC), xylazine from Bayer Canada (Etobicoke, ON), buprenorphine HCl from Reckitt Colman Pharmaceuticals (Richmond, VA) and heparin from Leo Laboratories Canada (Ajax, ON). Halothane BP was manufactured by Halocarbon Laboratories (Riveredge, NJ). The 5'-nucleotidase 15.5 reagent, bovine serum albumin, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulphonic acid (CHAPS), hippuryl-L-histidyl-L-leucine (HHL), *p*-nitrophenyl phosphate and phosphoramidon were from Sigma–Aldrich (Mississauga, ON). Aminopeptidase M and alkaline phosphatase-labeled anti-digoxigenin Fab fragments were from Boehringer Mannheim (Laval-des-Rapides, QC). Goat IgG and biotinylated goat anti-rabbit IgG were from Santa Cruz (Santa Cruz, CA) and the avidin-peroxidase complex was from Vector Lab. (Burlingame, CA). Ethanol of HPLC grade was purchased from American Chemicals Ltd. (Montréal, QC). The bicinchoninic acid protein assay procedure and heptafluorobutyric anhydride (HFBA) were from Pierce (Rockford, IL). 7-Amido-4-methyl coumarin (AMC) and succinyl-alanyl-alanyl-phenylalanyl-AMC (Suc-Ala-Ala-Phe-AMC) were from Bachem (Bubendorf, Switzerland). Acetonitrile (HPLC grade) and all other chemicals of analytical grade were obtained from Fisher Scientific (Montréal, QC). All BK amino-truncated peptides were synthesized using solid phase synthesis by Dr. G. Drapeau (Centre de Recherche, Hôtel-Dieu de Québec, Québec, Canada) (10).

3.2. Surgery and animal sacrifice

All the animal experiments followed the guidelines of the Canadian Council on Animal Care and were approved by the Animal Care Ethics Committee of the Institut de Cardiologie de Montréal. Myocardial infarction was induced in 200-250 g male Wistar rats (Charles River, St-Constant, QC) through ligation of the left descendant coronary artery as described earlier (3, 26). The rats were anesthetized with halothane 3%. During surgery they were artificially ventilated with humidified room air supplemented with oxygen and the halothane concentration was gradually decreased to 1%. A Harvard Rodent ventilator (Harvard Apparatus, South Natick, MA) set at 2 cc, 70 strokes/min and a Fluotec 3 halothane vaporizer (Cyprane, UK) were used for this procedure. The heart was quickly exteriorized through a left-sided thoracotomy and the left descendant coronary artery was ligated approximately 2 mm from its origin. The heart was then replaced in its normal position in the thorax and the incision was closed with a Mikron wound clip applicator (Clay Adams) after gently pressing the chest to expel air from the cavity to avoid a pneumothorax. Once awakening following surgery, the rats were injected with buprenorphine 0.01 to 0.02 mg/kg to reduce the pain during recovery.

On day 1, 4 and 35 after the surgery the surviving rats were sacrificed in order to obtain the heart. The rats were anesthetized using an intra-muscular injection of ketamine (50 mg/kg) and xylazine (10 mg/kg). Prior to the sacrifice, during the same anesthesia, an electrocardiogram (ECG) was performed and the intraventricular pressures were measured by inserting a Millar Mikro-Tip Catheter Transducer (Millar Instruments, Houston, TX) with a pressure sensor at the tip into both the jugular vein and carotid artery, and advancing it into the right and left ventricle, respectively. The ECG and pressures were recorded on a Gould 2,600S recorder (Gould Inc., Cleveland, OH). The rats were classified as having shammed, small, medium or large myocardial infarctions according to the ECG readings taken prior to their sacrifice. The presence of a Q wave in the lead I and lead AVL derivative signaled a myocardial infarction. The heights of the R waves in the V_1, V_2 and V_5 derivatives were summed and their total value was used as a criterion for further classification. A value inferior to 0.6 mV indicated a large infarction,

a value greater than 1.0 mV indicated a sham or small infarction and one between 0.6 mV and 0.85 mV indicated a medium infarction. Sham hearts and hearts with small infarctions were pooled and compared to the second group consisting of medium and large infarctions. Before removing the heart, 1000 units/kg of heparin were injected in the jugular vein. The heart was then excised and carefully perfused with a 37°C saline solution to remove all blood. Once this was completed, the infarcted hearts were dissected into two pieces: the infarcted area of the left ventricular wall (scar) and the remainder of the heart (the non-infarcted area). In this model, the infarcted area is not clearly visible 24 hours postinfarction and always involves the free wall of the left ventricle, and the septum is never implicated in the infarct. Hearts sampled 24 hours postinfarction had the left ventricular wall dissected free of the septum. The left ventricular wall was then considered the infarcted area and the septum as viable myocardium. All portions of the hearts were frozen at –80°C until used for biochemical investigations.

3.3. Preparation of the total heart membrane suspensions

To assess the metabolism of BK by enzymes located on cardiac cell membranes, membranes were extracted from the hearts following a procedure previously used by Kinoshita et al. (15) to study the metabolism of angiotensin I and more recently by Blais et al. (5) to assess the metabolism of BK in the normal rat heart. The non-infarcted and infarcted portions of each heart were thawed, weighed and then cut into 3- to 4-mm pieces. These pieces were placed in a 50 mM Tris-HCl buffer, pH 7.4, at 4°C (10 ml/g of tissue) and homogenized with a Polytron homogenizer (Brinkmann Instruments, Rexdale, ON) at setting 8 for 15 sec. The homogenate was centrifuged at 40,000 g for 20 minutes at 4°C. After centrifugation, the tissue pellet consisting of membranes was separated from the cytosolic supernatant. The membranes were resuspended in a 50 mM Tris-HCl buffer, pH 7.4, containing 100 mM NaCl, at 4°C. A Wheaton Potter-Elvehjem tissue grinder (Fisher Scientific, Pittsburgh, PA), driven by a T-line motorized stirrer (Talboys Engineering Corp., Emerson, NJ) turning at setting 8 for 60 seconds, was used for this

procedure. During resuspension, the fibrous tissue was discarded from the infarcted pieces. The resulting membrane suspension was assayed for its 5'-nucleotidase activity (1). The protein concentration of the membrane suspensions was determined by the bicinchoninic acid method using bovine serum albumin as the standard.

3.4. BK metabolism

3.4.1. Incubation of BK with the total heart membrane suspensions

The membrane suspensions were diluted with a 50 mM Tris-HCl buffer, pH 7.4, containing 100 mM NaCl, in order to obtain a 5 mg/ml protein concentration. The membrane suspensions coming from the infarcted scar pieces were pooled two by two on the basis of an equal protein concentration in order to obtain a sufficient suspension volume for the incubation procedure. The metabolic profile of BK was measured at 37°C in the same conditions previously used for normal heart (5). Briefly, 10 µl of saline containing 500 ng of synthetic BK were added to 990 µl of the heart membrane suspension. The final concentration of BK in this suspension was 471 nM. After various incubation periods at 37°C, ranging between 2 min and 20 min, the reaction was stopped by precipitating the membrane proteins through the addition of cold (4°C) ethanol at a final concentration of 80% vol/vol. In two sets of parallel experiments, and prior to adding the synthetic BK, the membrane suspensions were pre-incubated for 15 min at 37°C either with enalaprilat or with omapatrilat. The inhibitor concentrations were respectively 130 nM for enalaprilat and 510 nM for omapatrilat. The precipitated samples were centrifuged 15 min at 4°C and 2000 × g. The clear supernatant containing BK and its metabolites was evaporated to dryness in a Speed Vac Concentrator (Savant, Farmingdale, NY). The residues were stored at –80°C until quantification of the residual BK was performed.

3.4.2. Quantification of BK

Immunoreactive BK was quantified in the residues of the evaporated ethanolic extracts using a highly specific enzyme immunoassay developed in our laboratory (8). This assay used highly specific polyclonal rabbit IgG raised against the carboxy-terminal end of BK, digoxigenin-labeled peptide as tracer and alkaline phosphatase-labeled anti-digoxigenin Fab fragments with the substrate *p*-nitrophenyl phosphate to detect and quantify the immune complexes. Each sample was measured in triplicate. Typical calibration curves were characterized by half-maximal saturation values of 0.78 pmol/ml. This method was precise and accurate.

3.4.3. Kinetic parameters analysis

BK hydrolysis constant rate (k) was evaluated with the first-order equation $[BK]= [BK]_c \times e^{-kt}$, where [BK] is the concentration of BK at a given time and $[BK]_0$ is [BK] at time (t) = 0. The BK half-life ($t_{½}$) was represented as $t_{½} = \ln(2)/k$ (20). The different $t_{½}$ values were expressed for 1 mg of protein.

ACE relative activity was estimated from k with $[k_{(+)}]$ or without $[k_{(-)}]$ enalaprilat using the equation $ACE\% = 100 \times [1 - k_{(+)}/k_{(-)}]$. NEP relative activity was estimated by using k with $[k_{(+)}]$ or without $[k_{(-)}]$ omapatrilat and by subtracting the ACE relative activity, following the equation $NEP\% = \{100 \times [1 - k_{(+)}/k_{(-)}]\} - ACE\%$ (20). This subtraction assumed that both enalaprilat and omapatrilat inhibit ACE to the same extent. In fact, the concentration chosen for each inhibitor was above its K_i, so that both inhibitors fully inhibit ACE.

3.4.4. Separation and identification of BK amino-truncated metabolites

In order to assess if the immunoreactive BK measured at the $t_{½}$ corresponds to the native amino-terminal peptide, immunograms after HPLC chromatography were designed for each incubation condition (5). Briefly, in the conditions described above, BK was incubated with the heart membrane preparations for a period corresponding to the calculated $t_{½}$. Incubations were performed either in the presence of enalaprilat, in the presence of omapatrilat or without inhibitor. After precipitation of proteins with cold ethanol and centrifugation, the ethanolic extracts were separated in two parts. After evaporation, the first part was used for the quantification of immunoreactive BK. The second part was dissolved in 0.025% HFBA (vol/vol) in distilled water before HPLC separation. A HPLC system (Waters Associates, Milford, MA) consisting of a Model 600 Multisolvent Delivery System and a Model 484 Tunable Absorbance Detector was employed for HPLC analysis. BK and four products of the amino-terminal enzymatic cleavage of BK were separated on a reverse phase column (Vydac C_{18} 5 μm, 4.6 × 250 mm; Hesperia, CA) at a constant flow rate of 0.7 ml/min using a 45-min linear gradient from 80% solvent A/20% solvent B to 65% solvent A/35% solvent B. Solvent A was 0.025% HFBA (vol/vol) in distilled water and solvent B was 0.025% HFBA (vol/vol) in 90% acetonitrile/10% distilled water. The column effluent was monitored continuously at 214 nm. Fractions of 0.7 ml were collected, evaporated to dryness in a Speed Vac Concentrator and then frozen at –80°C until immunoreactivity profile determination. BK and metabolites were identified by comparing their retention times with those of reference peptides.

3.4.5. Measurement of ACE and NEP activity

The membrane suspensions used for BK metabolism were solubilized in 8 mM 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulphonic acid (CHAPS) (6). ACE activity was measured using the method of Cushman and Cheung (7) and NEP activity was measured using the method of Nortier et al. (22). Each sample was quantified in duplicate for both assays. ACE activity was expressed in pmol of hippuric acid/min/mg

of protein and NEP activity was expressed in pmol of 7-amido-4-methyl-coumarin (AMC)/min/mg of protein.

3.5. Immunohistochemistry of NEP and ACE expression

Expression of NEP and ACE were determined immunohistochemically. The hearts of the rats were fixed in a 10% formalin-phosphate buffered solution. The hearts were embedded in paraffin, sectioned (6 μm width) with a microtome along the cross-section of the specimen at the midpoint between the apex and the base of the heart and applied on glass slides. The sections were deparaffinized in xylene and ethanol baths and endogenous peroxidase activity was quenched in a methanol-hydrogen peroxide solution. A non-specific antibody binding was prevented by pre-incubating the tissues with a 5% goat serum treatment. Sections were exposed to the primary antibodies of rabbit polyclonal anti-rat NEP IgG (1:500 dilution) raised and purified in our laboratory (P. Crine, unpublished observations) and monoclonal anti-rat ACE IgM (1:500 dilution) kindly provided by Dr. R. Auerbach (University of Wisconsin, Madison, WI) and used according to his recommandations (2). A purified non-specific goat IgG (1:500 dilution; Santa Cruz, Santa Cruz, CA) was used as a primary negative control. The secondary antibodies were biotinylated goat anti-rabbit IgG (1:400 dilution; Vector Lab., Burlingame, CA) and goat anti-mouse IgM (1:100 dilution; Vector Lab.), respectively. Revelation of bound antibodies was achieved with an avidin/peroxidase complex (Vector Lab.) and counter-stained in Gill's hematoxylin solution. NEP and ACE expression (brown staining) was evaluated for each segment by using a dedicated 3CCD video microscope adapted to a customized software.

3.6. Statistical analysis

All data are expressed as means ± standard error of the mean (SEM). Different models of analysis of variance were used to analyze the data. Two-way factorial analysis with days

and tissues, sham and non-infarcted or infarcted and non-infarcted were used with Scheffé's contrasts. A two-way analysis with the factor days and a repeated factor tissues (infarcted and non-infarcted) was also used with paired t-tests using Bonferroni inequality. Finally, in some cases a one-way analysis with appropriate contrasts was used. In view of the multiple analysis performed on the data the significance level was fixed at 1%. Since most of the analysis of variance showed interaction, only the results of the analysis of contrasts are reported.

4. Results

4.1. Hemodynamic characteristics

Hearts with a myocardial infarction (MI) had a significant increase in left ventricular end-diastolic pressure (LVEDP). On day 1, MI hearts had a LVEDP of 9 ± 2 mm Hg, on day 4 a LVEDP of 9 ± 1 mm Hg and on day 35 a LVEDP of 7 ± 2 mm Hg, all these pressures were significant ($P < 0.01$) as compared to sham-operated controls (day 1: 1 ± 2 mm Hg; day 4: 3 ± 1 mm Hg; day 35: 1 ± 2 mm Hg, respectively).

4.2. Effect of MI on the metabolism of BK

The $t_{½}$ of exogenous BK measured in cardiac membranes from the sham hearts was consistent throughout the five weeks study period, at between 114 ± 7 to 143 ± 6 sec (Fig. 1, page 68). The $t_{½}$ in the viable portion of the infarcted hearts was similar to that of sham at one day postinfarction (107 ± 8 sec; n = 21 and 123 ± 6 sec; n = 10, respectively), but was significantly decreased by four days (85 ± 4 sec; n = 20 in MI versus 114 ± 7 sec; n = 11 in sham; $P < 0.01$). BK was thus metabolized 1.3 times faster in the viable portion of the MI hearts as compared to shams. However by 35 days postinfarction, the $t_{½}$ had returned to levels similar to those of the sham group (137 ± 9 sec; n = 14 and 143 ± 6 sec; n = 10, respectively). The infarcted portion of the heart had no difference in BK $t_{½}$ as compared to sham or viable myocardium of MI hearts one day postinfarction. By 4 days postinfarction, BK $t_{½}$ was markedly decreased, to 28 ± 2 sec (n = 10), levels that were approximately 33% of those of sham and 25% of those of the non-infarcted portion of the same hearts ($P < 0.01$). By 35 days postinfarction, BK $t_{½}$ in the infarcted region had nearly doubled (55 ± 4 sec; n = 7) as compared to four days postinfarction (28 ± 2 sec; n = 10; $P < 0.01$), but remained less than half of that of the non-infarcted portion of the same hearts and of sham hearts ($P < 0.01$). BK was thus metabolized 2.5 faster in the

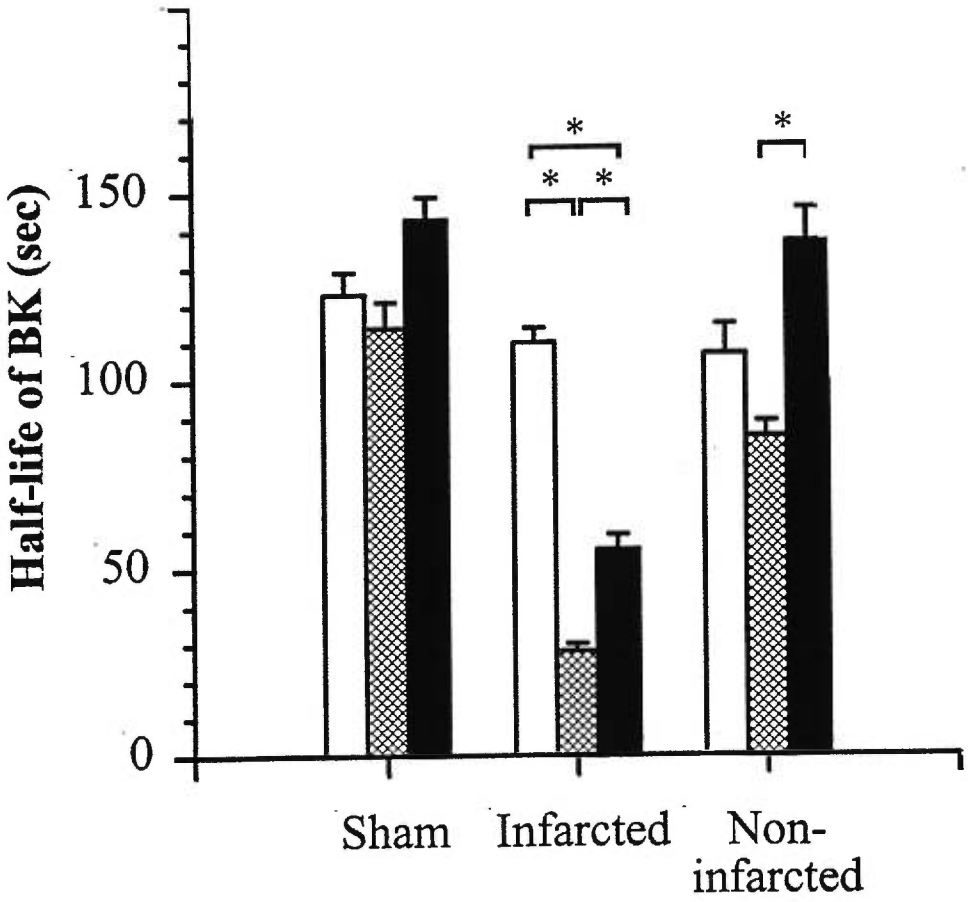

Figure 1
Time course of changes in the half-life of exogenous BK incubated with heart membranes from sham hearts, and the infarcted zone and non-infarcted zone of infarcted hearts removed 1 (open columns), 4 (crosshatched columns) and 35 (closed columns) days post-myocardial infarction. Data are means ± SEM; * $P < 0.01$.

infarcted region at 35 days as compared to sham or the viable myocardium of these hearts.

4.3. Effect of enalaprilat and omapatrilat on BK half-life ($t_{½}$)

The pre-incubation of the membrane preparations with enalaprilat increased BK $t_{½}$ significantly ($P < 0.01$) in every tissue type and at every sampling time following surgery (Fig. 2, page 70). Omapatrilat increased BK $t_{½}$ significantly even more than did enalaprilat ($P < 0.01$) (Fig. 2, page 70). In sham hearts, the effect of both inhibitors remained unchanged over time (Fig. 3, page 71). However, in the infarcted and non-infarcted portions of the MI hearts the effects of both inhibitors increased over time ($P < 0.01$) (Fig. 3, page 71). In infarcted pieces, the effect of both inhibitors on BK $t_{½}$ was the greatest at 35 days postinfarction (163 ± 8 sec for enalaprilat and 199 ± 14 sec for omapatrilat; n = 7) as compared to day 1 (36 ± 8 sec and 68 ± 7 sec, respectively; n = 11) ($P < 0.01$) or day 4 (35 ± 4 sec and 43 ± 4 sec, respectively; n = 10) ($P < 0.01$) following infarction. In the non-infarcted pieces, an increase in effect of both inhibitors on BK $t_{½}$ occured earlier, at 4 days postinfarction (126 ± 7 sec for enalaprilat and 185 ± 12 sec for omapatrilat; n = 20) versus 1 day postinfarction (63 ± 10 sec and 97 ± 14 sec, respectively; n = 21) ($P < 0.01$). By 35 days postinfarction, the increase in effect of enalaprilat on BK $t_{½}$ in non-infarcted portions of MI hearts was no longer significant as compared to day 1. However, the increase in effect of omapatrilat on BK $t_{½}$ as compared to day 1 was maintained up to 35 days (168 ± 14 sec; n = 14) versus day 1 (97 ± 14 sec; n = 21) ($P < 0.01$).

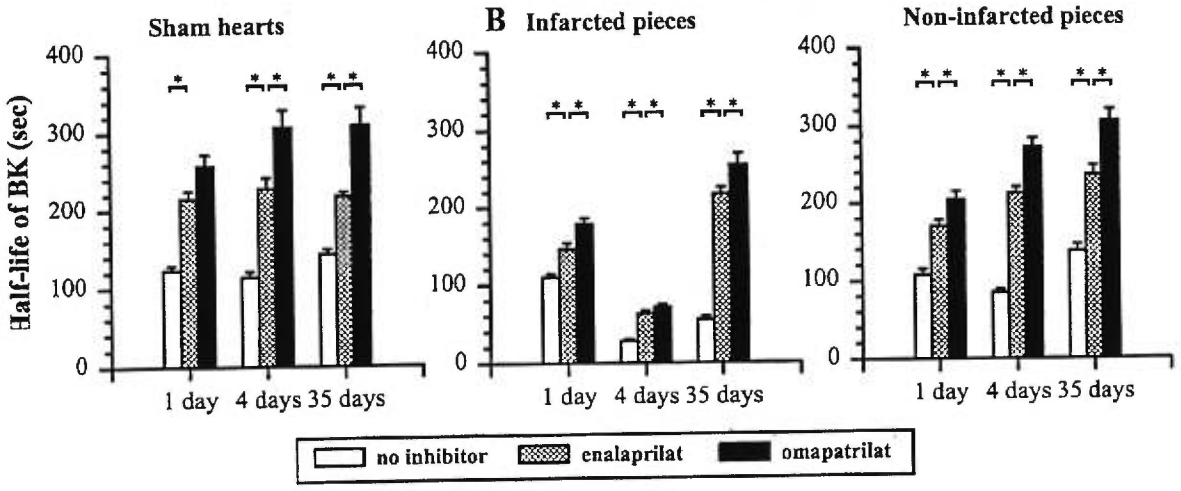

Figure 2

Half-life of exogenous BK incubated with heart membranes from sham hearts (*A*), and the infarcted zone (*B*) and non-infarcted zone (*C*) of infarcted hearts removed 1, 4 and 35 days post-myocardial infarction. The membranes were preincubated without inhibitor (open columns), with enalaprilat (crosshatched columns) or with omapatrilat (closed columns). Data are means ± SEM; * $P < 0.01$.

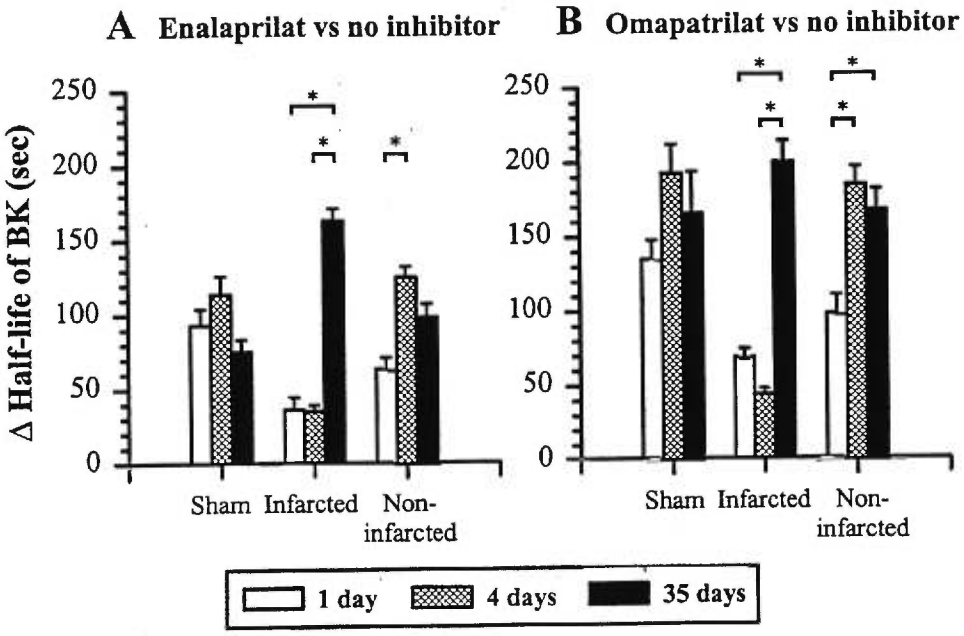

Figure 3

Difference in half-life of exogenous BK between treatments with enalaprilat and without inhibitor (*A*), and treatments with omapatrilat and without inhibitor (*B*). BK was incubated with membranes from sham hearts, and the infarcted zone and non-infarcted zone of infarcted hearts removed 1 (open columns), 4 (crosshatched columns) and 35 (closed columns) days post-myocardial infarction. Data are means ± SEM; * $P < 0.01$.

4.4. Relative contribution of ACE and NEP on BK metabolism

The effect of enalaprilat on the BK $t_{½}$ was used as a measure of the involvement of ACE in the metabolism of BK. The difference between the effects of omapatrilat and enalaprilat was used as a reflection of the involvement of NEP in this metabolism (Fig. 4, page 73). In sham hearts, the additive effect of NEP on BK metabolism remained unchanged through the study period. In the non-infarcted pieces of MI hearts the involvement of NEP increased gradually over time such that it was significantly higher ($P < 0.01$) by 35 days postinfarction (69 ± 7 sec; n = 14) as compared to 1 day postinfarction (34 ± 6 sec; n = 21). In infarcted pieces, NEP inhibition significantly prolonged the $t_{½}$ of BK 1 day postinfarction (32 ± 5 sec; n = 11), however, by 4 days postinfarction this was no longer true (8 ± 1 sec; n = 10). The effect of NEP inhibition on BK $t_{½}$ was re-established by 35 days postinfarction (37 ± 9 sec; n = 7).

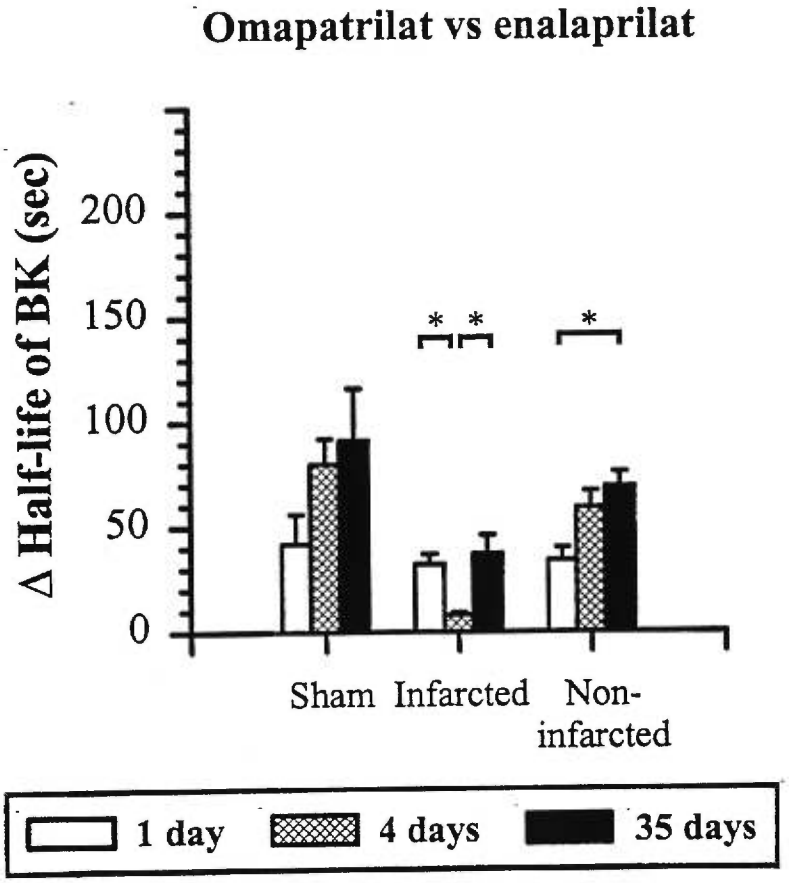

Figure 4
Difference in half-life of exogenous BK between treatments with omapatrilat and with enalaprilat. The BK was incubated with membranes from sham hearts, and the infarcted zone and non-infarcted zone of infarcted hearts removed 1 (open columns), 4 (crosshatched columns) and 35 (closed columns) days post-myocardial infarction. Data are means ± SEM; * $P < 0.01$.

If one adjusts the additive effects of ACE and NEP inhibition to BK $t_{1/2}$ without drugs, the relative contribution of ACE and NEP on BK metabolism in the various tissues at any given point in time can be calculated (see Table I). The relative importance of ACE and NEP on the metabolism of BK varies according to the nature of the tissue and over time. When compared to NEP, ACE played a greater role in the metabolism of BK in every tissue and at every time point evaluated. In the sham hearts, the relative participation of ACE remained unchanged over time. In the infarcted pieces the relative participation of ACE to the metabolism of BK was similar at day 1 postinfarction as compared to sham, however it rose steadily over time more than tripling between days 1 and 35 ($P < 0.01$), such that at day 35 it was twice that of sham ($P < 0.01$). In the non-infarcted pieces, the relative participation of ACE was similar to that of sham on day 1 postinfarction. It then rose from day 1 to day 4 postinfarction ($P < 0.01$), then returned to basal levels by day 35. The relative importance of NEP in sham or non-infarcted pieces of myocardium was similar at day 1 postinfarction and did not vary over time. In the infarcted pieces, the relative participation of NEP was similar at day 1 postinfarction as compared to the other two tissues, but it decreased over time ($P < 0.01$) such that it was lower than the sham hearts by day 4 and 35 postinfarction ($P < 0.01$).

	Relative participation of ACE (%)			Relative participation of NEP (%)		
	Day 1	Day 4	Day 35	Day 1	Day 4	Day 35
Sham	42.1 ± 3.4	49.2 ± 3.0	34.0 ± 3.5	9.2 ± 2.8	12.5 ± 1.6	17.1 ± 3.6
Infarcted	22.4 ± 4.9	54.3 ± 3.1*	74.8 ± 1.7*†	15.1 ± 3.0	5.3 ± 1.1*	3.5 ± 0.8*
Non-infarcted	35.9 ± 4.2	59.4 ± 1.8*	41.5 ± 3.0†	9.0 ± 1.6	8.4 ± 1.0	12.5 ± 1.3

Table I : Relative participation of angiotensin-converting enzyme (ACE) and neutral endopeptidase (NEP) in the hydrolysis of exogenous BK incubated with different heart pieces. Values are mean ± SEM. * $P < 0.01$ vs day 1; † $P < 0.01$ vs day 4.

4.5. Evolution of ACE and NEP enzymatic activities over time

Both ACE and NEP enzymatic activity exhibited important variations according to the nature of the tissue and its sampling time (Fig. 5, page 76). In the sham hearts, ACE activity remained unchanged over time from 164 ± 8 pmol/min/mg of protein (n = 9) on day 1 to 156 ± 6 pmol/min/mg of protein (n = 9) on day 35. NEP activity was similar at day 1 and day 4 (24.9 ± 1.9 pmol/min/mg of protein; n = 10 and 25.4 ± 1.9 pmol/min/mg of protein; n = 11, respectively), and it decreased to 17.1 ± 1.8 pmol/min/mg of protein (n = 9) on day 35, but it did not reach statistical significance ($P > 0.01$).

In the infarcted pieces from MI hearts, the ACE activity was similar on day 1 (120 ± 11 pmol/min/mg of protein, n = 15) as compared to sham. It then increased progressively over time such that by 4 days postinfarction the highest values for any tissue was recorded (357 ± 19 pmol/min/mg of protein, n = 8) ($P < 0.01$). By 35 days postinfarction, ACE activity (338 ± 15 pmol/min/mg of protein; n = 8) remained similar to day 4. The activity of NEP was higher in the infarcted pieces as compared to sham on the three time points ($P < 0.01$). Moreover, NEP activity was similar after 1 and 4 days, but it decreased to at day 35 (41.7 ± 3.7 pmol/min/mg of protein; n = 7) as compared to day 4 (70.7 ± 5.7 pmol/min/mg of protein; n = 4) ($P < 0.01$).

In the non-infarcted pieces from MI hearts, ACE activity was higher on day 1 than in sham ($P < 0.01$), but it remained unchanged over time from 205 ± 17 pmol/min/mg of protein (n = 20) on day 1 to 143 ± 15 pmol/min/mg of protein (n = 14) on day 35. NEP activity was lower at day 4 (31.2 ± 1.9 pmol/min/mg of protein; n = 17) and day 35 (30.8 ± 4.2 pmol/min/mg of protein; n = 10) as compared to day 1 (45.4 ± 2.7 pmol/min/mg of protein; n = 17) ($P < 0.01$).

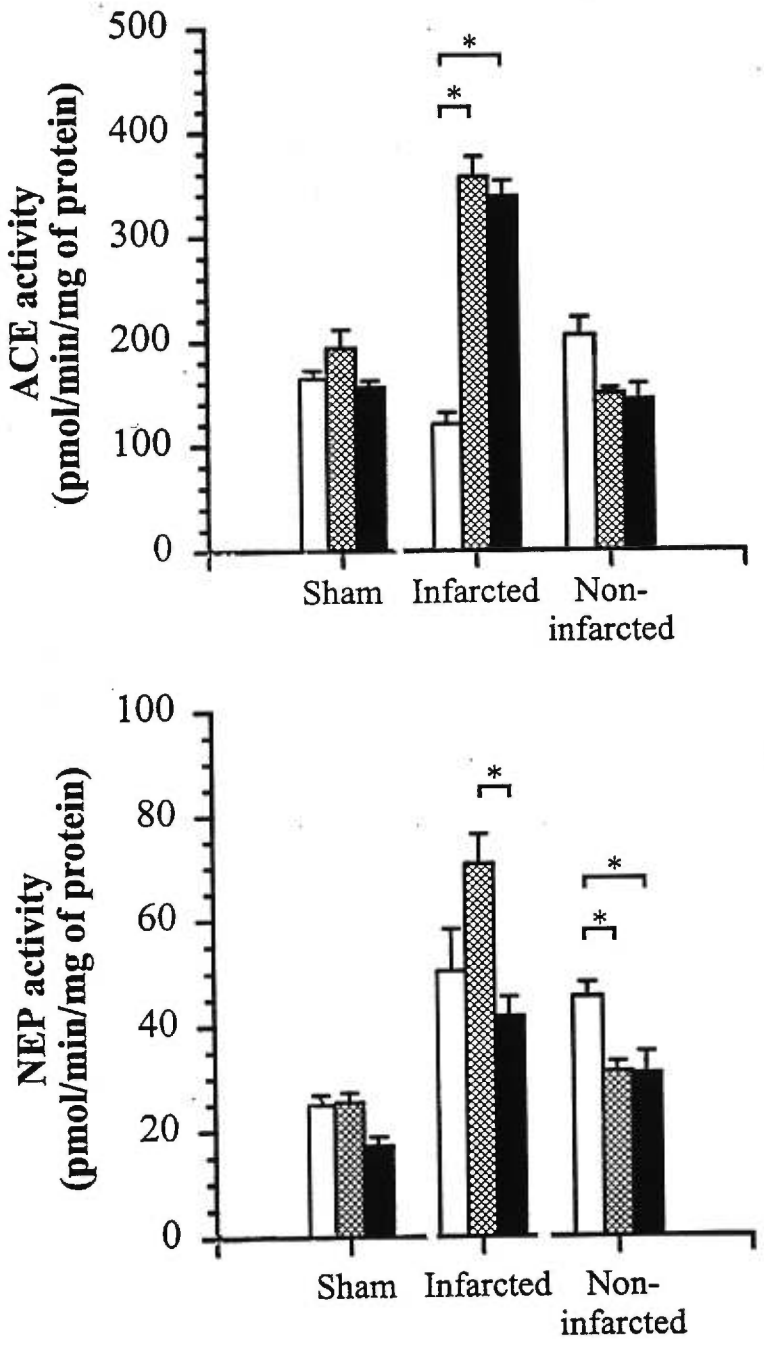

Figure 5

Angiotensin-converting enzyme (ACE) activity (*A*) and neutral endopeptidase (NEP) activity (*B*) of the membranes from sham hearts, and the infarcted zone and non-infarcted zone of infarcted hearts removed 1 (open columns), 4 (crosshatched columns) and 35 (closed columns) days post-myocardial infarction. Data are means ± SEM; * $P < 0.01$.

4.6. Amino-terminal metabolism of BK by the heart membrane preparations

HPLC analysis performed with residual BK after incubation with membranes coming from 4 days infarcted pieces, indicated that the immunoreactive BK measured at the $t_{1/2}$, in the absence and in the presence of enalaprilat or omapatrilat (Fig. 6, page 78), corresponds to the native peptide. In fact, more than 95% of the detected immunoreactive BK corresponds to the non-amino-truncated B_2 agonist. Similar recovery values were measured for sham hearts and the non-infarcted parts of the infarcted hearts.

4.7. Protein expression of NEP and ACE in normal and hypertrophied hearts

In absence of cardiac injury in normal hearts, we could not detect the expression of NEP immunohistochemically with an antibody that specifically recognizes the expression of this protein (Fig. 7D, page 79). We also analyzed the expression of NEP on the hearts of rats with a myocardial infarction at 35 days post-procedure. In the non-infarcted region of the heart, we could not detect the expression of NEP (Fig. 7E, page 79), however, in the infarcted area of the heart, we observed a clear and localized expression of NEP in some of the cardiomyocytes (Fig. 7F, page 79). In each study, a purified non-specific goat IgG was used as a primary negative control, and in each case we could not detect any positive staining (Fig. 7A-C, page 79). In a similar manner, we also evaluated the expression of ACE. In all hearts (normal and infarcted), we observed an ubiquitous expression of ACE on cardiomyocytes and endothelial cells (Fig. 8D-F, page 80). In each study, a purified non-specific goat IgG was used as a primary negative control, and in each case we could not detect any positive staining (Fig. 8A-C, page 80).

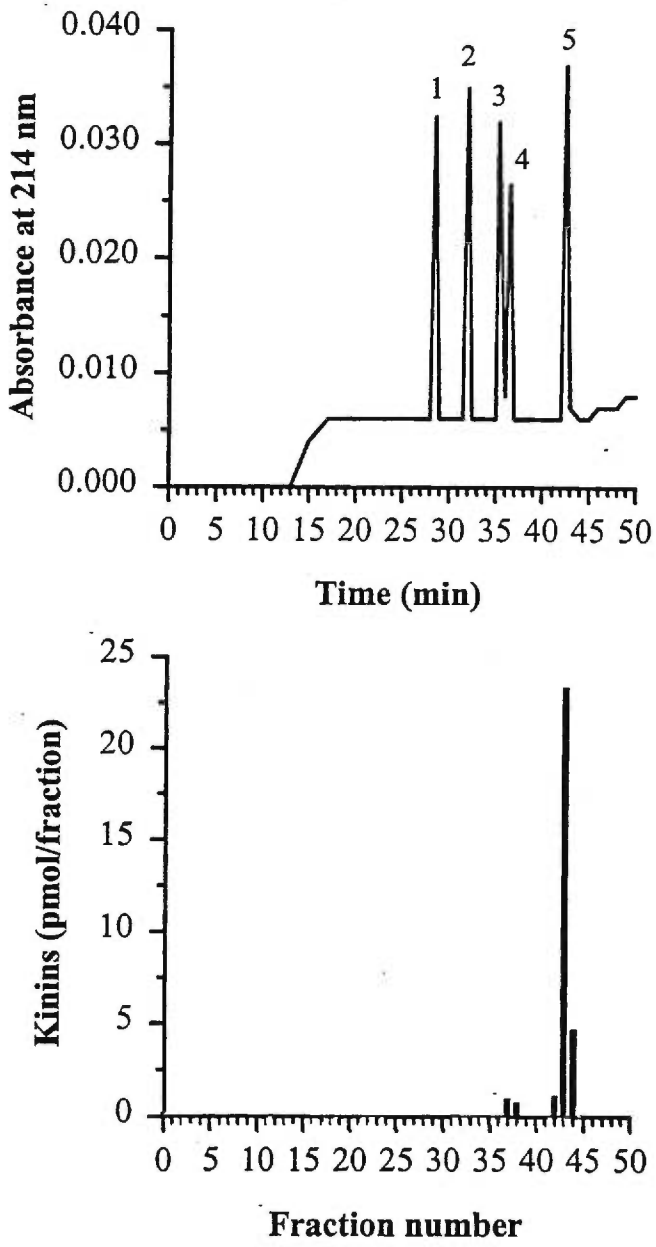

Figure 6

A: retention times for reference peptides of amino-terminal truncated metabolites of BK: BK-(5–9), 28.5 min (*1*); BK-(4–9), 32.0 min (*2*); BK-(3–9), 35.3 min (*3*); BK-(2–9), 36.5 min (*4*); and BK, 42.3 min (*5*). *B*: immunoreactivity profile after reverse-phase high-performance liquid chromatography of incubation medium after BK was incubated with membranes from the infarcted area of infarcted heart taken at day 4 in the presence of omapatrilat. Extraction and chromatographic conditions are described in Materials and Methods.

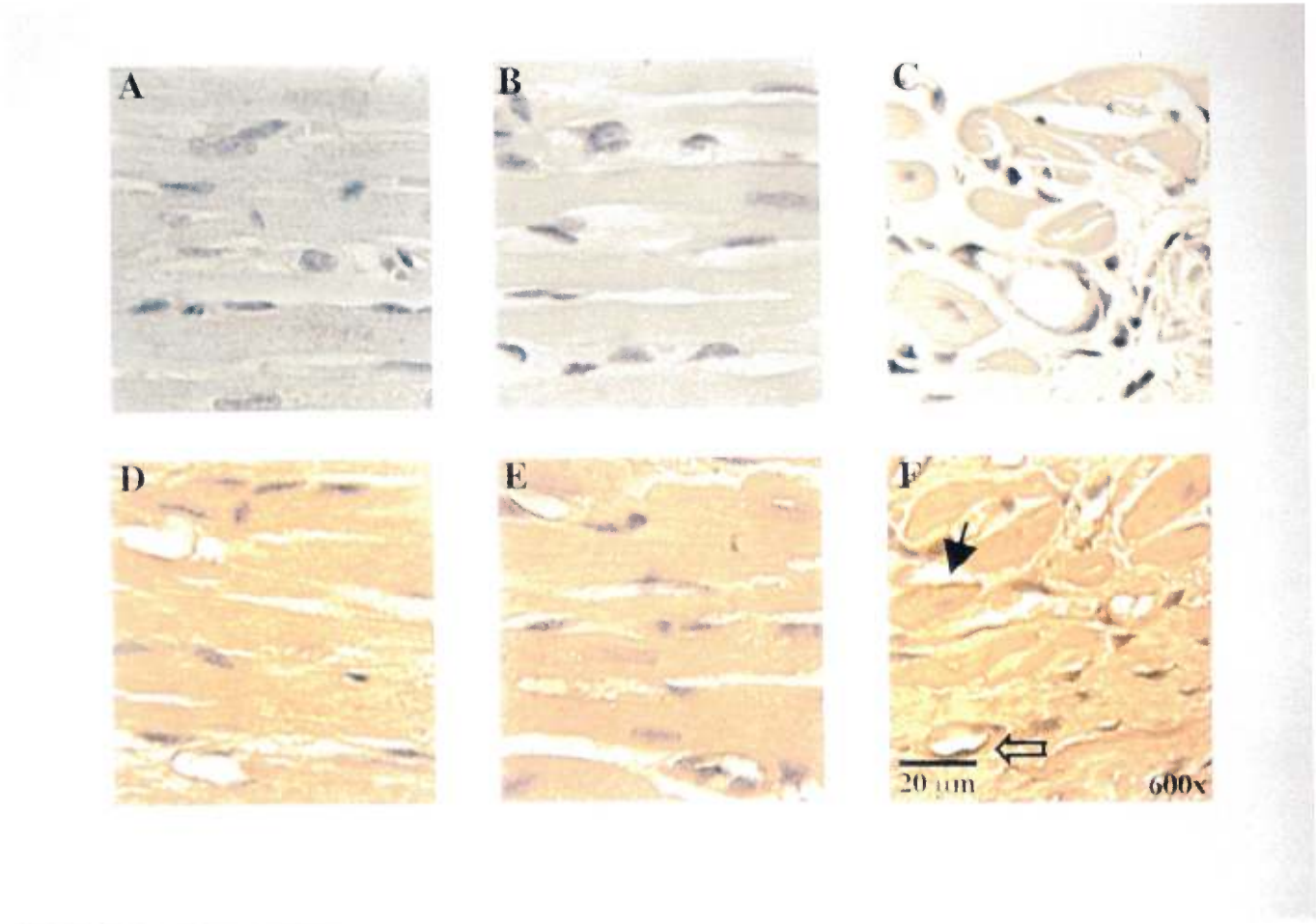

Figure 7

Neutral endopeptidase 24.11 (NEP) expression on representative cross-sections of control and infarcted hearts (35 days postinfarction): in absence of primary antibodies (*A, B, C*) and in the presence of primary antibodies (*D, E, F*), in control heart (A, D), infarcted heart (non-infarcted region) (*B, E*) and the infarcted region (scarred) (*C, F*). Positive NEP expression on cardiomyocytes is revealed by brown staining (closed arrow). Magnification × 600. Immunohistochemistry protocol is described in Materials and Methods.

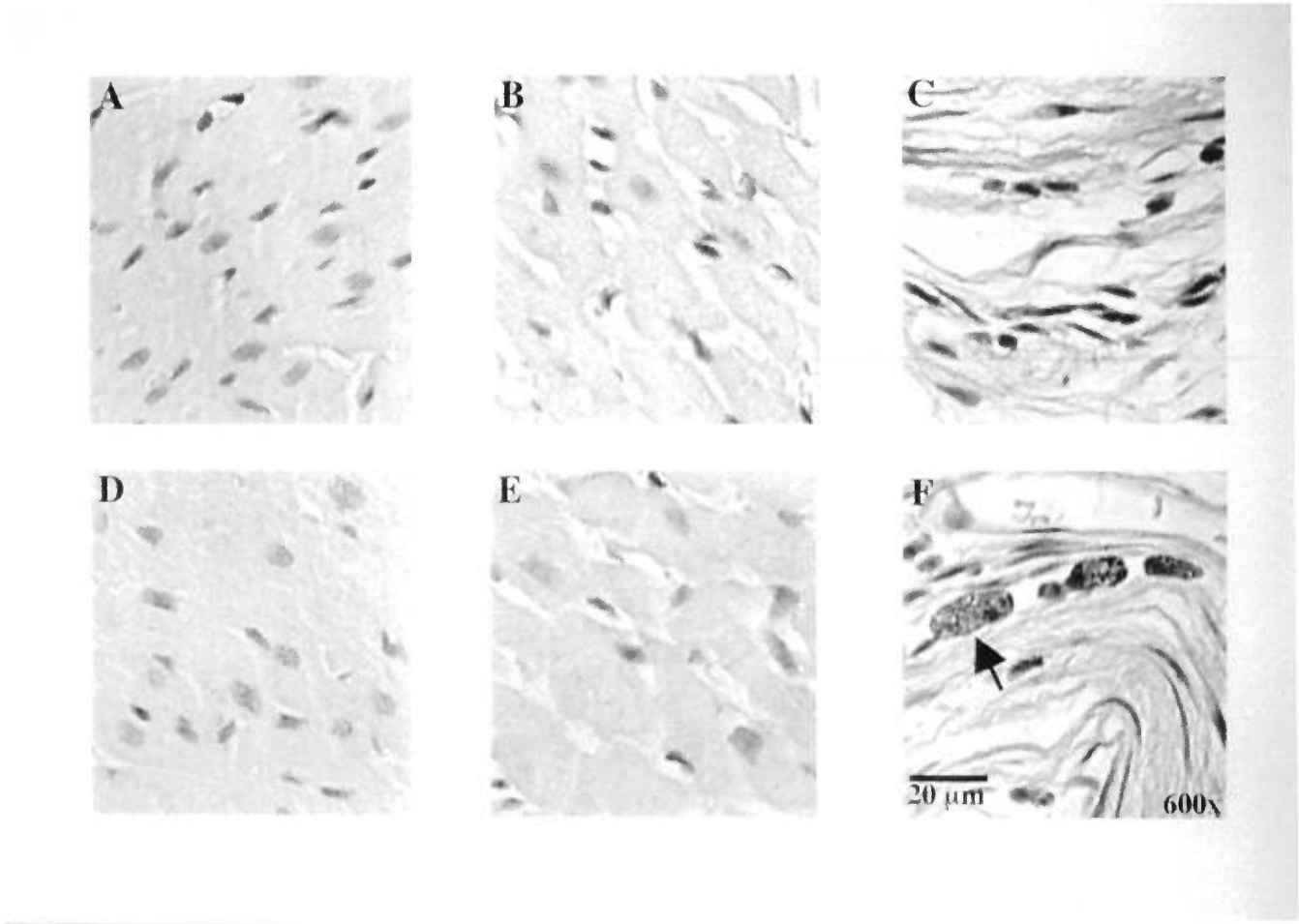

Figure 8

Angiotensin-converting enzyme (ACE) expression on representative cross-sections of control and infarcted hearts (35 days postinfarction): in absence of primary antibodies (*A, B, C*) and in the presence of primary antibodies (*D, E, F*), in control heart (A, D), infarcted heart (non-infarcted region) (*B, E*) and the infarcted region (scarred) (*C, F*). Positive ACE expression is revealed by brown staining on cardiomyocytes (closed arrow) and endothelial cells (opened arrow). Magnification × 600. Immunohistochemistry protocol is described in Materials and Methods.

Discussion

In this study, we demonstrated for the first time an alteration in the metabolism of BK by cardiac membranes during the acute, subacute, and chronic postinfarction periods. These alterations varied according to the time postinfarction and the tissue (infarcted or non-infarcted) being studied. Moreover, we showed clearly that an ACE inhibitor and even more so the new vasopeptidase inhibitor, omapatrilat, a dual ACE/NEP inhibitor, prevents the degradation of this vasodilatory and anti-proliferative peptide at each time point and in each tissue. Again these effects varied according to the tissue and the time point postinfarction and, at each time of our experimental protocol, the effect of omapatrilat was significantly more important than that of enalaprilat on the degradation of BK not only by sham and non-infarcted tissues but also by the scar.

The rat postinfarction model has been widely used to study the pathophysiologic processes involved in acute and chronic postinfarction left ventricular remodeling (23, 24). In the early postinfarction, there is an acute inflammatory process in the area of the scar that involves cardiac necrosis and infiltration by numerous inflammatory cell types. This inflammatory process intensifies within the next few days, then, gradually disappears as a fibrotic scar develops. The non-infarcted area is under significant hemodynamic stress and intense neurohumoral stimulation during the acute and subacute postinfarction periods (23, 24, 29). Later postinfarction, hemodynamic stress and neurohumoral activation abates in those in which beneficial compensatory ventricular remodeling occurs. A number of studies suggest that BK may play a critical role in promoting beneficial remodeling and that the cardioprotective effects of ACE inhibitors in this setting are largely due to their prolongation of BK $t_{½}$ (17, 19). In those where adverse ventricular remodeling occurs or MI size is very large, hemodynamic stress and neurohumoral activation as well as low level inflammation, as reflected by a rise in systemic cytokines, persists. In this study, mild to moderate left ventricular dysfunction accompanied the induction of the MI suggesting at least partial compensatory ventricular remodeling.

In the heart, endogenous kinins can be produced locally during a MI by two mechanisms. First, as the heart possesses an independent kallikrein-kinin system (21), they can originate from the tissue itself. Second, BK can also originate from plasma. Indeed, in a previous clinical study, we have shown a decrease of prekallikrein and plasma kininogens in the postinfarction period in man (A. Adam, Thèse de l'agrégation de l'enseignement supérieur, Université de Liège, Belgium). In this paper, we applied to the postinfarction period the same experimental in vitro metabolic approach we have used to define the metabolism of BK in the normal heart of different animal species (5). We clearly showed that MI significantly shortens the $t_{½}$ of BK. In the infarcted zone, this decrease only becomes evident 4 days postinfarction, a time at which the infarcted zone is scarring and the acute inflammatory response is still important. It also persists until at least 35 days postinfarction a time at which the inflammatory response has largely abated. In the non-infarcted portions of the MI hearts, BK $t_{½}$ also decreases, although it does so only at 4 days postinfarction and returns to the level of sham by 35 days postinfarction, a period where hypertrophy of the remaining viable heart is well established.

As BK was incubated with membrane preparations and $t_{½}$ was expressed per mg of total membrane proteins, the metabolic changes documented cannot be attributed to an artefactual dilution factor, nor to soluble intracellular or extracellular enzymes modified during and after MI, but rather to enzyme activities modified at the plasmatic cell membrane level. Membrane preparations used in this protocol are representative of normal cardiac muscle as they are composed of at least 75% cardiomyocyte membranes (36). However, we cannot exclude that membranes prepared from infarcted and hypertrophied samples contain membranes of cells other than cardiomyocytes. That is particularly true 4 days post-MI when infiltrating inflammatory cells (neutrophils, macrophages and fibroblasts) are at their peak in the necrotic zone (33) and could release cytokines that could upregulate the enzyme activity in an autocrine or paracrine way (16).

Preincubation of membranes with an ACE inhibitor significantly increased the $t_{½}$ of BK in the different tissue samples evaluated. The potentiating effect of an ACE inhibitor was similar in sham and non-infarcted tissues. In the infarcted portion of the heart however, the effect of ACE inhibitor on the BK $t_{½}$ was less important 1 and 4 days post-MI. Four days post-MI, BK $t_{½}$ was markedly decreased in these membranes, and preincubation with enalaprilat did not succeed in normalizing $t_{½}$. In these infarcted pieces, the effect of the ACE inhibitor was mainly evident 35 days postinfarction a time at which hypertrophy of the remaining viable myocardium has developed. At that time, ACE inhibitor increased BK $t_{½}$ four-fold. These results are consistent with those of Johnston et al. (14) who, using a quantitative autoradiographic method, showed an increased ACE expression in rat heart 4 weeks postinfarction. This increase was particularly important in the fibrous scar tissue of the infarcted area. However, when the relative participation of ACE in the metabolism of BK is calculated from the $t_{½}$ values in presence and in absence of ACE inhibitor, the values of both kinetic parameters show clearly that, although important in the metabolism of BK, ACE is not the only enzyme responsible for the inactivation of BK and that the relative participation of these other enzymes varies according to the tissue and timing postinfarction. Differences in the activation of other enzymes (5, 15) would thus explain the only incomplete correlation between ACE activity and the relative contribution of ACE inhibitor on BK metabolism.

Among the other enzymes potentially responsible for the degradation of BK, NEP must be considered as a serious candidate. Recently, in defining the metabolism of BK by the coronary vascular bed of normal rat heart, we have shown that coperfusion of retrothiorphan, a highly specific NEP inhibitor, with BK did not modify its metabolism. However, when perfused in the presence of enalaprilat, retrothiorphan significantly increased the recovery of BK by 36% when compared with enalaprilat alone (11). These in vitro results with normal coronary endothelium show clearly that when ACE is inhibited, NEP takes over and plays an important role in the metabolism of BK. The behaviour of both enzymes can be explained by their respective affinity for BK. These results constitute the experimental basis for the simultaneous inhibition of both enzymes

by a vasopeptidase inhibitor that exhibits similar inhibitory potency for both ACE (IC_{50} = 5 nM) and NEP (IC_{50} = 9 nM). Our results obtained using cardiac membranes in normal and pathological hearts not only confirm but also extend these previous observations made at the normal coronary endothelium level.

When heart membranes were preincubated with omapatrilat, an increase in BK $t_{½}$ occurred, which was greater than the one measured in presence of the ACE inhibitor. Although omapatrilat reduced the metabolism of BK in all evaluated tissues, the simultaneous inhibition of NEP and ACE was particularly effective in prolonging BK $t_{½}$ in the infarcted portion of the heart 1 day post-MI. At that time, the relative participation of NEP in the degradation of BK averaged that of ACE. This observation suggests that the use of omapatrilat may be particularly beneficial early in the postinfarction period, when BK appears to be involved in the regulation of the acute inflammatory reaction that stabilizes the scar. Although still significant, the protective effect of omapatrilat on BK was less important in the infarcted zone at 4 and 35 days postinfarction and in the non-infarcted zone, as well as in sham hearts, at all time-points measured. The superiority of omapatrilat over a simple ACE inhibitor in reducing the degradation of BK could explain some of the experimental data recently obtained in vivo which could involve the effects of endogenous BK.

In the rat, inhibition of NEP protected the heart against ischemia/reperfusion injury, as evidenced by a significant reduction of MI size and a tendency towards a reduction of reperfusion arrhythmias (38). These effects of NEP inhibitors were blocked by the B_2 receptor antagonist icatibant suggesting that the accumulation of kinins participates in the cardioprotective effect of NEP inhibitors. However, as NEP is also involved in the metabolism of natriuretic peptides, it has been shown that natriuretic peptides could be responsible, at least in part, for the cardiovascular effects of these new vasopeptidase inhibitors (28, 31, 34). In another study, it was reported that inhibitors of NEP and endopeptidase 24.15 produced cardioprotective effects comparable to those of ramipril in a rabbit model of ischemia/reperfusion (30). It was also demonstrated that a combination

of ACE and endopeptidase inhibition produced more protection than when either type inhibitor was used alone. Trippodo et al. (34) reported that in hamsters with cardiomyopathy, the combined inhibition of ACE and NEP caused a significant decrease in left ventricular pressure and total peripheral vascular resistance and an increase in cardiac output compared to vehicle or either ACE inhibitor and NEP inhibitor alone. Finally, omapatrilat has been found to prolong the survival of cardiomyopathic hamsters more than did an ACE inhibitor, suggesting superior cardioprotective effects in this model of left ventricular dysfunction (accepted for AHA presentation).

We also measured the enzyme activity of ACE and NEP, however the values measured are difficult to put in relation with the $t_{½}$ of BK or the relative participation of both these metallopeptidases. Indeed, when we inhibit both enzymes, we do not totally block the degradation of BK (Fig. 9, page 86). This was particularly evident 4 days postinfarction in the scar. In the infarcted part of the heart, as well as in the non-infarcted part of the heart and in the sham heart, we cannot exclude the induction of other membrane enzymes capable of metabolizing BK, by the cytokines released at the local inflammatory site or activated systemically. Moreover, we do not know the nature, the properties and the importance of these enzymes which account for the rest of BK metabolism in each tissue sample.

Finally, using a polyclonal antibody directed against rat NEP, we could find evidence of the presence of this enzyme in cardiomyocytes in the infarcted border zone 35 days postinfarction in amounts greater than in sham myocardium suggesting that NEP is particularly active in this area at that time period. Moreover, immunohistochemical analysis using an anti-ACE antibody allowed us to confirm the presence of ACE not only in the endothelium, but also in cardiomyocytes (14, 33).

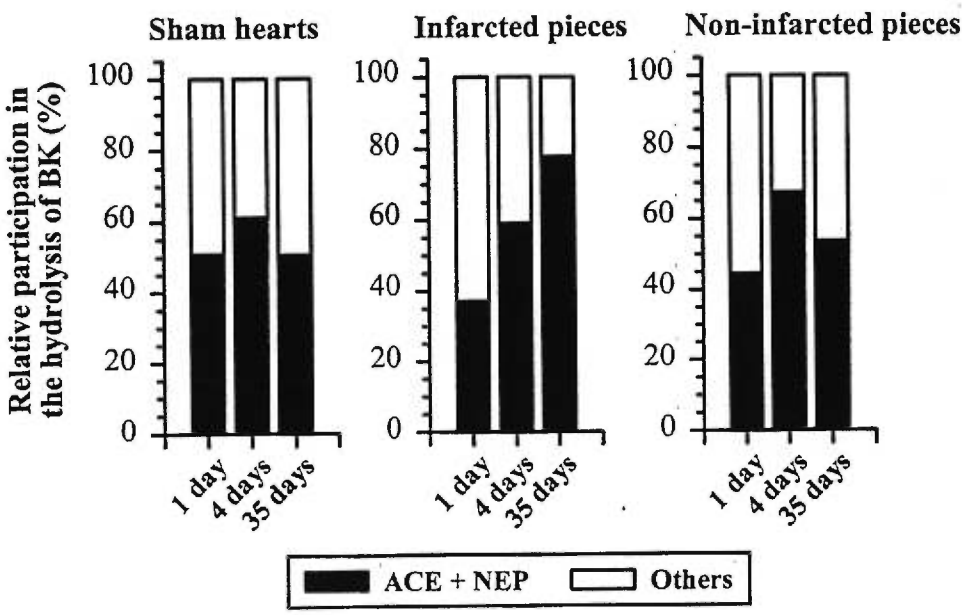

Figure 9
Synoptic representation of relative contribution of ACE + NEP to the metabolism of BK.

Our results on the effects of an ACE inhibitor and the vasopeptidase inhibitor omapatrilat on the metabolism of BK may have pathophysiological significance and can be used as a rational basis for further studies in the acute and chronic postinfarction setting. As the heart contains the precursors of BK and their tissue activator (21), our observations for exogenous BK may be transposed to endogenous BK. However, in that case, not only the membrane enzymes must be considered, but also the circulating enzymes released from the membranes and cytosol during ischemia.

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6. References

1. **Arkesteijn, C. L. M.** A kinetic method for serum 5'-nucleotidase using stabilized glutamate dehydrogenase. *J. Clin. Chem. Clin. Biochem.* 14: 155-158, 1976.

2. **Auerbach, R., L. Alby, J. Grieves, J. Joseph, C. Lindgren, L. W. Morrissey, Y. A. Sidky, M. Tu, and S. L. Watt**. Monoclonal antibody against angiotensin-converting enzyme: its use as a marker for murine, bovine, and human endothelial cells. *Proc. Natl. Acad. Sci. USA*. 79: 7891-7895, 1982.

3. **Bélichard, P., P. Savard, R. Cardinal, R. Nadeau, H. Gosselin, P. Paradis, and J. L. Rouleau.** Markedly different effects on ventricular remodeling result in a decrease in inducibility of ventricular arrhytmias. *J. Am. Coll. Cardiol.* 23: 505-513, 1994.

4. **Bhoola, K. D., C. D. Figueroa, and K. Worthy.** Bioregulation of kinins: kallikreins, kininogens and kininases. *Pharmacol. Rev.* 44: 1-80, 1992.

5. **Blais, C., Jr., G. Drapeau, P. Raymond, D. Lamontagne, N. Gervais, I. Venneman, and A. Adam.** Contribution of angiotensin-converting enzyme to the cardiac metabolism of bradykinin: an interspecies study. *Am. J. Physiol.* 273 (*Heart Circ. Physiol.* 42): H2263-H2271, 1997.

6. **Costerousse, O., J. Allegrini, H. Huang, J. Bounhik, and F. Alhenc-Gelas.** Regulation of ACE gene expression and plasma levels during rat postnatal development. *Am. J. Physiol.* 267 (*Endocrinol. Metab.* 30): E745-E753, 1994.

7. **Cushman, D. W., and H. S. Cheung.** Spectrophotometric assay and properties of the angiotensin-converting enzyme of rabbit lung. *Biochem. Pharmacol.* 20: 1637-1648, 1971.

8. **Décarie, A., G. Drapeau, J. Closset, R. Couture, and A. Adam.** Development of digoxigenin-labeled peptide: application to chemiluminoenzyme immunoassay of bradykinin in inflamed tissues. *Peptides* 15: 511-518, 1994.

9. **Décarie, A., P. Raymond, N. Gervais, R. Couture, and A. Adam.** Serum interspecies differences in metabolic pathways of bradykinin and [des-Arg^9]BK: influence of enalaprilat. *Am. J. Physiol.* 270 (*Heart Circ. Physiol.* 39): H1340-H1347, 1996.

10. **Drapeau, G., and D. Regoli.** Synthesis of bradykinin analogs. *Methods Enzymol.* 163: 263-272, 1988.

11. **Dumoulin, M.-J., A. Adam, C. Blais, Jr., and D. Lamontagne.** Metabolism of bradykinin by the rat coronary vascular bed. *Cardiovasc. Res.* 38: 229-236, 1998.

12. **Erdös, E. G., and R. A. Skidgel.** Metabolism of bradykinin by peptidases in health and disease. In: *The Kinin System*, edited by S. G. Farmer. San Diego, CA: Academic Press, 1997, p. 111-141.

13. **Hall, J. M.** Bradykinin receptors: pharmacological properties and biological roles. *Pharmacol. Ther.* 56: 131-190, 1992.

14. **Johnston, C. I., V. Mooser, Y. Sun, and B. Fabris.** Changes in cardiac angiotensin converting enzyme after myocardial infarction and hypertrophy in rats. *Clin. Exp. Pharmacol. Physiol.* 18: 107-110, 1991.

15. **Kinoshita, A., H. Urata, F. M. Bumpus, and A. Husain.** Measurement of angiotensin I converting enzyme inhibition in the heart. *Circ. Res.* 73: 51-60, 1993.

16. **Kondepudi, A., and A. Johnson.** Cytokines increase neutral endopeptidase activity in lung fibroblasts. *Am. J. Respir. Cell Mol. Biol.* 8: 43-49, 1993.

17. **Linz, W., and B. A. Schölkens.** Role of bradykinin in the cardiac effects of angiotensin-converting enzyme inhibitors. *J. Cardiovasc. Pharmacol.* 20 (Suppl. 9): S83-S90, 1992.

18. **Linz, W., G. Wiemer, P. Gohlke, T. Unger, and B. A. Schölkens.** Contribution of kinins to the cardiovascular actions of angiotensin-converting enzyme inhibitors. *Pharmacol. Rev.* 47: 25-49, 1995.

19. **Liu Y.-H., X.-P. Yang, V. G. Sharov, O. Nass, H. N. Sabbah, E. Peterson, and O. A. Carretero.** Effects of angiotensin-converting enzyme inhibitors and angiotensin II type 1 receptor antagonists in rats with heart failure. Role of kinins and angiotensin II type 2 receptors. *J. Clin. Invest.* 99: 1926-1935, 1997.

20. **Moore, J. W., and R. G. Pearson.** Complex reactions. In: *Kinetics and Mechanism* (3rd ed.), edited by J. W. Moore and R. G. Pearson. New York: Wiley, 1981, p. 284-296.

21. **Nolly, H., L. A. Carbini, G. Scicli, O. A. Carretero, and A. G. Scicli.** A local kallikrein-kinin system is present in rat hearts. *Hypertension* 23 [part 2]: 919-923, 1994.

22. **Nortier, J., S. Pauwels, E. De Prez, and M. Deschodt-Lanckman.** Human neutrophil and plasma endopeptidase 24.11: quantification and respective roles in atrial natriuretic peptide hydrolysis. *Eur. J. Clin. Invest.* 25: 206-212, 1995.

23. **Pfeffer, J. M.** Progressive ventricular dilation in experimental myocardial infarction and its attenuation by angiotensin-converting enzyme inhibition. *Am. J. Cardiol.* 68: 17D-25D, 1991.

24. **Pfeffer, M. A., and E. Braunwald.** Ventricular remodeling after myocardial infarction: experimental observations and clinical implications. *Circulation* 81: 1161-1172, 1990.

25. **Pfeffer, M. A., E. Braunwald, L. A. Moyé, L. Basta, E. J. Brown, T. E. Cuddy, B. R. Davis, E. M. Geltman, S. Goldman, G. C. Flaker, M. Klein, G. A. Lamas, M. Packer, J. Rouleau, J. L. Rouleau, J. R. Rutherford, J. H. Wertheimer, and C. M. Hawkins.** Effect of captopril on mortality and morbidity in patients with left ventricular dysfunction after myocardial infarction: results of the survival and ventricular enlargement trial. *N. Engl. J. Med.* 327: 669-677, 1992.

26. **Pfeffer, M. A., J. M. Pfeffer, M. C. Fishbein, P. J. Fletcher, J. Spadaro, R. A. Kloner, and E. Braunwald.** Myocardial infarct size and ventricular function in rats. *Circ. Res.* 44: 503-512, 1979.

27. **Proud, D., and K. P. Kaplan.** Kinin formation: mechanisms and role in inflammatory disorders. *Ann. Rev. Immunol.* 6: 49-83, 1988.

28. **Robl, J. A., C.-Q. Sun, J. Stevenson, D. E. Ryono, L. M. Simpkins, M. P. Cimarusti, T. Dejneka, W. A. Slusarchyk, S. Chao, L. Stratton, R. N. Misra, M. S. Bednarz, M. M. Asaad, H. S. Cheung, B. E. Abboa-Offei, P. L. Smith, P. D. Mathers, M. Fox, T. R. Schaeffer, A. A. Seymour, and N. C. Trippodo.** Dual metalloprotease inhibitors: mercaptoacetyl-based fused heterocyclic dipeptide mimetics as inhibitors of angiotensin-converting enzyme and neutral endopeptidase. *J. Med. Chem.* 40: 1570-1577, 1997.

29. **Rouleau, J. L., J. de Champlain, M. Klein, D. Bichet, L. Moyé, M. Packer, G. R. Dagenais, B. Sussex, J. M. Arnold, F. Sestier, J. O. Parker, P. McEwan, V. Bernstein, T. E. Cuddy, G. Lamas, S. S. Gottlieb, J. McCans, C. Nadeau, F. Delage, P. Hamm, and M. A. Pfeffer.** Activation of neurohumoral systems in postinfarction left ventricular dysfunction. *J. Am. Coll. Cardiol.* 22: 390-398, 1993.

30. **Schriefer, J. A., E. P. Broudy, and A. H. Hassen.** Endopeptidase inhibitors decrease myocardial ischemia/reperfusion injury in an in vivo rabbit model. *J. Pharmacol. Exp. Ther.* 278: 1034-1039, 1996.

31. **Seymour, A. A., M. M. Asaad, B. E. Abboa-Offei, P. L. Smith, W. L. Rogers, and C. R. Dorso.** In vivo pharmacology of dual neutral endopeptidase/angiotensin-converting enzyme inhibitors. *J. Cardiovasc. Pharmacol.* 28: 672-678, 1996.

32. **Skidgel, R. A.** Basic carboxypeptidases: regulators of peptide hormone activity. *Trends Pharmacol. Sci.* 9: 299-304, 1988.

33. **Sun, Y., J. P. M. Cleutjens, A. Diaz-Arias, and K. T. Weber.** Cardiac angiotensin converting enzyme and myocardial fibrosis in the rat. *Cardiovasc. Res.* 28: 1423-1432, 1994.

34. **Trippodo, N. C., M. Fox, V. Natarajan, B. C. Panchal, C. R. Dorso, and M. M. Asaad.** Combined inhibition of neutral endopeptidase and angiotensin converting enzyme in cardiomyopathic hamsters with compensated heart failure. *J. Pharmacol. Exp. Ther.* 267: 108-116, 1993.

35. **Webb, R. L., M. L. Abramson, M. E. Beil, L. M. Odorico, and R. E. Chatelain.** Effects of the novel dual inhibitor of neutral endopeptidase and angiotensin-converting enzyme, CGS 30440, on blood pressure and cardiac hypertrophy in spontaneously hypertensive rats. *J. Cardiovasc. Pharmacol.* 30: 632-642, 1997.

36. **Weber, K. T., and C. G. Brilla.** Pathological hypertrophy and cardiac interstitium. Fibrosis and renin-angiotensin-aldosterone system. *Circulation* 83: 1849-1865, 1991.

37. **Wollert, K. C., R. Studer, K. Doerfer, E. Schieffer, C. Holubarsch, H. Just, and H. Drexler.** Differential effects of kinins on cardiomyocyte hypertrophy and interstitial collagen matrix in the surviving myocardium after myocardial infarction in the rat. *Circulation* 95: 1910-1917, 1997.

38. **Yang, X.-P., Y.-H. Liu, E. Peterson, and O. A. Carretero.** Effect of neutral endopeptidase 24.11 inhibition on myocardial ischemia/reperfusion injury: the role of kinins. *J. Cardiovasc. Pharmacol.* 29: 250-256, 1997.

Chapter 3:

DISCUSSION

In this study we have defined, for the first time, the influence of myocardial infarction and left ventricular hypertrophy on the metabolism of exogenous BK. For this purpose, we used a clinically relevant animal model, whereby myocardial infarction was induced *in vivo* in the rat by the ligature of the anterior descending coronary artery. The metabolism of exogenous BK was then characterized *in vitro* using an experimental approach, which has previously been used to study the metabolism of Ang I and that of BK in normal hearts from different animal species. We have shown for the first time that myocardial infarction modifies the half-life of BK. This was particularly true 4 days post-infarction, when a decrease in BK half-life could be measured not only in the infarcted area, but also in the non-infarcted one. This decrease was still significant 36 days post-infarction, at which time myocardial hypertrophy was established.

Using specific inhibitors, we also evidenced an important participation of ACE, as well as of NEP, in the cardiac metabolism of BK. These results were confirmed by two alternative experimental approaches, namely the quantification of enzyme activity using synthetic substrates and the immunolocalization of both enzymes in the myocardium using highly specific antibodies. We have also discovered that the participation of both ACE and NEP in the metabolism of BK varies with the time-course of the disease and with the nature of the tissue, be it infarcted or non-infarcted. These enzymes moreover are not the only ones responsible for the cardiac degradation of BK. In fact, neither the preincubation of the cardiac membranes with an ACEi, nor that with a dual vasopeptidase inhibitor succeeded in completely blocking the metabolism of the B_2 agonist. This was particularly true 4 days post-infarction and we cannot exclude the induction of other membrane enzymes capable of metabolizing BK, by the cytokines released at the local inflammatory site or activated systemically.

Our results are important from several points of view. First, they demonstrate for the first time that the importance of ACE in the cardiac metabolism of BK may be altered by heart disease. Although ACEi have been successfully used for over 25 years in the treatment of acute and chronic heart conditions, the importance of ACE in the cardiac metabolism of BK had never been documented in the post-myocardial infarction period. Moreover, our

results show that the participation of ACE varies according to the time-course of heart disease. This observation could be important, given the debate over whether or not ACEi treatment should be started early after myocardial infarction. ACE however is not the only enzyme responsible for the cardiac metabolism of BK. NEP also plays an important role in this metabolism, once ACE has been inhibited. Our observations constitute an objective and experimental basis for the development of dual vasopeptidase inhibitors as well as for their therapeutic use following infarction, which aims among others things, to achieve an improved protective effect on BK over mere ACE ihibition. Finally, the *in vitro* experimental approach used in this project is part of a broader study under way in our lab. In this study, we are defining the *in vivo* effect of a chronic dual vasopeptidase treatment on the level of endogenous BK in the infarcted heart, as well as in the hypertrophied cardiac tissue. The levels on BK are then related to hemodynamic, morphometric and biochemical parameters used to assess myocardial infarct size and the extent of the left ventricular hypertrophy.

REFERENCES

Acker GR, Molineaux C and Orlowski M. Synaptosomal membrane-bound form of endopeptidase 24.15 generates Leu-enkephalin from dynorphin$_{1-8}$, α- and β-neoendorphin, and Met-enkephalin from Met-enkephalin-Arg^6-Gly^7-Leu^8. *Journal of Neurochemistry* 48: 284-292, 1987.

Alhenc-Gelas F, Soubrier F, Hubert C, Allegrini J, Lattion AL and Corvol P. The angiotensin I-converting enzyme (kininase II): progress in molecular and genetic structure. *Journal of Cardiovascular Pharmacology* 15(suppl 5): S25-S29, 1990.

Antman ME and Braunwald E. Acute myocardial infarction. In "*Heart Disease*" (ed Braunwald E) 5th ed, ch 37 p1184-1288 W.B. Saunders Company, Philadelphia. 1997.

Asano M, Hatori C, Sawai H, Johki S, Inamura N, Kayakiri H, Satoh S, Abe Y, Inoue T, Sawada Y, Mitzutani T, Oku T and Nakahara K. Pharmacological characterization of a nonpeptide bradykinin antagonist, FR165649 and agonist FR190997. *British Journal of Pharmacology* 124(3):441-446, 1998.

Aubert JF, Waeber B, Nussberger J, Vavrek R, Stewart JM and Brunner HR. Lack of a role of circulating bradykinin in the blood pressure response to acute angiotensin converting enzyme inhibition in rats. *Agents and Actions* 22: 312-317, 1993.

Barnes K, Ingram J and Kenny AJ. Proteins of the kidney microvillar membrane. Structural and immunochemical properties of rat endo peptidase-2 and its immunohistochemical localization in tissues of rat and mouse. *Biochemical Journal* 264: 335-346, 1989.

Bascands JL, Pecher C, Bompart G, Rakotoaryvony J, Leung-Tack J and Girolami JP. Bradykinin-induced in vitro contraction of rat mesangial cells via a B2 receptor type. *American Journal of Physiology* 267: F871-F878, 1994.

Bascands JL and Girolami JP. La Bradykinine. *Médecine/Sciences* 12:582-92, 1996.

Beierwaltes WHP and Carretero OA. Effect of glandular kallikrein on renin release in isolated rat glomeruli. *Hypertension* 7:27-31, 1985.

Bhoola KD, Figueroa CD and Worthy K. Bioregulation of kinins: kallikreins, kininogens and kininases. *Pharmacological Reviews* 44(1): 1-80, 1992.

Blais C Jr, Drapeau G, Raymond P, Lamontagne D, Gervais N, Venneman I and Adam A. Contribution of angiotensin-converting enzyme to the cardiac metabolism of bradykinin: an interspecies study. *American Journal of Physiology*, 273(5Pt2):H2263-H2271, 1997.

Blais C Jr, Rouleau JL, Brown NJ, Lepage Y, Spence D, Munoz C, Friborg J, Geadah D, Gervais N and Adam A. Serum metabolism of bradykinin and des-Arg^9-bradykinin in patients with angiotensin-converting enzyme inhibitor-associated angioedema. *Immunopharmacology* (in press), 1999a.

Blais C Jr, Marc-Aurèle J, Simmons WH, Loute G, Thibault P, Skidgel RA and Adam A. Des-Arg9-bradykinin metabolism in patients who presented hypersensitivity reactions during hemodialysis: role of serum ACE and aminopeptidase P. *Peptides* (in press), 1999b.

Blum A and Miller H. Role of cytokines in heart failure. *American Heart Journal* 135: 181-186, 1998.

Bönner G. The role of kinins in the antihypertensive and cardioprotective effects of ACE inhibitors. *Drugs* 54(Suppl 5): 23-30, 1997.

Borges DR and Gordon AH. Kininogen amd kininogenase synthesis by the liver of normal and injured rats. *Journal of Pharmacy and Pharmacology* 28(1):44-48, 1976.

Bralet J, Marie C, Mossiat C, Lecomte JM, Gros C and Scwartz JC. Effects of alatriopril, a mixed inhibitor of atriopeptidase and angiotensin-1 converting enzyme, on cardiac hypertrophy and hormonal responses in rats with myocardial infarction. Comparison with captopril. *Journal of Pharmacology and Experimental Therapeutics* 270:8-14, 1994.

Brosnihan KB, Li P and Ferrario CM. Angiotensin-(1-7) dilates canine coronary arteries through kinins and nitric oxide. *Hypertension* 27:523-528, 1996.

Brown NJ and Vaughan DE. Angiotensin-converting enzyme inhibitors. *Circulation* 97(14): 1411-1420, 1998.

Brown NJ, Blais C Jr, Gandhi SK and Adam A. ACE insertion/deletion genotype affects bradykinin metabolism. *Journal of Cardiovascular Pharmacology* 32:373-377, 1998.

Campbell DJ, Kladis A and Duncan AM. Differential effects of converting enzyme inhibition on angiotensin and bradykinin peptides in rat kidney and heart. *Ricerca Scientifica ed Educazione Permanente* (Milan) Suppl. 95:102, 1993.

Colucci WS. Molecular and cellular mechanisms of myocardial failure. *American Journal of Cardiology* 80(11A):15L-25L, 1997.

Colucci WS and Braunwald E. Pathophysiology of heart failure. In "*Heart Disease*" (ed Braunwald E) 5th ed, ch 13 p394-420 W.B. Saunders Company, Philadelphia. 1997.

Corvol P and Williams TA. Biochemical and molecular aspects of angiotensin I-converting enzyme. In "*Cell-Surface Peptidases in Health and Disease*" (eds. Kenny AJ and Boustead CM), 1997, pp 99-118. Bios Scientific Publishers, Oxford.

Craig SS, Reckelhoff JF and Bond JS. Immunohistochemical localization of the metalloproteinase meprin in salivary glands of male and female mice. *Journal of Histochemistry and Cytochemistry* 39: 123-129, 1991.

Cyr M, Gervais N, Blais C Jr, Hume H, Sweeney JD and Adam A. Anomaly of the des-Arg^9-bradykinin metabolism associated with severe hypotensive reactions during blood transfusions: a preliminary report. *Transfusion* (in press), 1999.

Danckwardt L, Shimizu I, Bonner G, Rettig R and Unger T. Converting enzyme inhibition in kinin-deficient Brown Norway rats. *Hypertension* 16(4):429-435, 1990.

Décarie A, Raymond P, Gervais n, Couture R and Adam A. Serum interspecies differences in metabolic pathways of bradykinin and [des-Arg^9]BK: Influence of enalaprilat. *American Journal of Physiology* 270:H1340-H1347, 1996.

Décarie A. Rôle des kinines et de leur récepteurs dans l'inflammation locale et systémique induite par la caragénine. Thèse de Doctorat. Département de physiologie, Faculté de Médecine, Université de Montréal, 1997.

Deddish PA, Skidgel RA, Kriho VB, Li XY, Becker RP and Erdös EG. Carboxypeptidase M in cultured Madin-Darby canine kidney (MDCK) cells. Evidence that carboxypeptidase M has a phosphatidylinositol glycan anchor. Journal of Biological *Chemistry* 265: 15083-15089, 1990.

DeLa Cadena RA and Colman RW. Structure and functions of human kininogens. *Trends in Biochemical Sciences* 12:272-275, 1991.

Dixon BS, Breckon R, Fortune J, Sutherland E, Simon RF and Anderson RJ. Bradykinin activates protein kinase C in cultured rat cortical collecting tubulular cells. *American Journal of Physiology* 257: F808-F817, 1989.

Dragovic T, Igic R, Erdös EG and Rabito AF. Metabolism of bradykinin by peptidases in the lung. *American Review of Respiratory Diseases* 147: 1491-1496, 1993.

Drummer OH, Kourtis S and Johnson H. Effect of chronic enalapril treatment on enzymes responsible for the catabolism of angiotensin I and the formation of angiotensin II. *Biochemical Pharmacology* 39:513-518, 1990.

Dumoulin MJ, Adam A, Blais C Jr and Lamontagne D. Metabolism of bradykinin by the rat coronary vascular bed. *Cardiovascular Research* 38:229-236, 1998.

Erdös EG. Kininases. In "*Bradykinin, Kallidin and Kallikrein. Handbook of Experimental Pharmacology*" (ed. Erdös EG), pp 427-487. Springer-Verlag, Heidelberg, 1979.

Erdös EG and Skidgel RA. Neutral endopeptidase 24.11-enkephalinase and related regulators of peptide hormones. *FASEB Journal* 3: 145-151, 1989.

Erdös EG, Wagner B, Harbury CB, Painter RG, Skidgel RA and Fa XG. Down-regulation and inactivation of neutral endopeptidase 24.11 (enkephalinase) in human neutrophils. *Journal of Biological Chemistry* 264(24): 14519-14523, 1989.

Erdös EG. Some old and some new ideas on kinin metabolism. *Journal of Cardiovascular Pharmacology* 15(suppl 6):S20-S24, 1990.

Erdös EG and Skidgel RA. Metabolism of bradykinin by peptidases in health and disease. In "*The Kinin System*". Farmer SG, Ed. Academic Press, San Diego, CA, 1997. P111-142.

Farhy RD, Ho KL, Carretero OA and Scicli AG. Kinins mediate the antiproliferative effect of ramirpril in rat carotid artery. *Biochemical and Biophysical Research Communications* 182:283-288, 1992.

Fahry RD, Carretero OA, Ho KL, et al. Role of kinins in the nitric oxide effects of angiotensin converting enzyme inhibitors on neointima formation. *Circulation Research* 72:1202-1210, 1993.

Falkenhahn M, Frenke F, Bohle RM, Zhu YC, Strauss HM, Bachmann S, Danilov S and Unger T. Cellular distribution of angiotensin-converting enzyme after myocardial infarction. *Hypertension* 25: 219-226, 1995.

Ferro CJ, Spratt JC, Haunes WG and Webb DJ. Inhibition of neutral endopeptidase causes vasoconstriction of human resistance vessels in vivo. *Circulation* 97(23):2323-2330, 1998.

Figueroa CD, Henderson LM, Colman RW, DeLa Cadena RA, Müller-Esterl W and Bhoola KD. Immunovisualisation of high (HK) and low (LK) molecular weight kininogens on isolated human neutrophils. *Blood* 79:754-759, 1988.

Figueroa CD, Maciver AG, Mackenzie JC and Bhoola KD. Localisation of immunoreactive kininogen and tissue kallikrein in the human nephron. *Histochemistry* 89:437-442, 1992.

French JF, Anderson BA, Downs TR and Dage RC. Dual inhibition of angiotensin-converting enzyme and endopeptidase in rats with hypertension. *Journal of Cardiovascular Pharmacology* 26:107-113, 1995.

Gabbiani G, Baddonel MC and Majno G. Vascular leakage induced by intra-arterial histamine, serotonin or bradykinin. *Proceedings of the Society for Experimental Biology and Medicine* 135: 447-452, 1970.

Gafford JT, Skidgel RA, Erdös EG and Hersh LB. Human kidney "enkephalinase", a neutral metallo-endopeptidase that cleaves active peptides. *Biochemistry* 22: 3265-3271, 1983.

Galjart NJ, Gillemans N, Meijer D and d'Azzo A. Mouse "protective protein" cDNA cloning, sequence comparison and expression. *Journal of Biological Chemistry* 265: 4678-4684, 1990.

Gibbons GH. Cardioprotective mechanisms of ACE inhibition: The Angiotensin II – Nitiric Oxide Balance. *Drugs* 54(S5):1-11, 1997.

Gohlke P, Linz W, Schölkens BA, Kuwer I, Bartenbach S, Schnell A and Unger T. Angiotensin-converting enzyme inhibition improves cardiac function: Role of bradykinin. *Hypertension* 23 : 411-418, 1994.

Gonzales-Vera W, Fournié-Zaluski MC, Pham I, Laboulandine I, Roques BP and Michel JB. Hypotensive and natriuretic effects of RB 105, a new dual inhibitor of angiotensin converting enzyme and neutral endopeptidase in hypertensive rats. *Journal of Pharmacology and Experimental Therapeutics* 272 :343-351, 1995.

Gustafson EJ, Schutsky D, Knight LC and Schmaier AH. High molecular weight kininogen binds to unstimulated platelets. *Journal of Clinical Investigation* 78: 310-318, 1986.

Gustafson EJ, Schmaier AH, Wachtvogel YT, Kaufman N, Kulich U and Colman RW. Human neutrophils contain and bind high molecular weight kininogen. *Journal of Clinical Investigation* 84: 28-35, 1989.

Hall JM. Bradykinin receptors: pharmacological properties and biological roles. *Phamacology and Therapeutics* 56:131-190, 1992.

Hall JM and Morton IKM. The Pharmacology and immunopharmacology of kinin receptors. In "*The Kinin System*". Farmer SG, Ed. Academic Press, San Diego, CA, 1997. P9-43.

Harbeck HT and Mentlein R. Aminopeptidase P from rat brain. Purification and action on bioactive peptides. *European Journal of Biochemistry* 198:451-458, 1991.

Hess JF, Derrick AW, MacNeil T and Borkowski JA. The agonist selectivity of a mouse B1 bradykinin receptor differs from human and rabbit B1 receptors. *Immunopharmacology* 33:1-8, 1996.

Hess JF. Molecular Pharmacology of Kinin Receptors. In "*The Kinin System*". Farmer SG, Ed. Academic Press, San Diego, CA, 1997. P45-55.

Hock FJ, Wirth K, Albus U, Linz W, Gerhards HJ, Wiemer G, Henke St, Breipohl G, König W and Knolle J. Hoe 140, a new potent and long acting bradykinin-antagonist : *in vitro* studies. *British Journal of Pharmacology* 102 :769-773, 1991.

Hooper NM, Hryszko J and Turner AJ. Purification and characterization of pig kidney aminopeptidase P. A glycosyl-phosphatidylinositol-anchored ectoenzyme. *Biochemical Journal* 267: 509-515, 1990.

Hooper NM, Hryszko J, Oppong SY and Turner AJ. Inhibition by converting enzyme inhibitors of pig kidney aminopeptidase P. *Hypertension* 19: 281-285, 1992.

Inamura N, Asano M, Kayakiri H, Hatori C, Oku T and Nakahara K. Characterization of FRI 173657 a novel non-peptide BK_2 antagonist: in vitro and in vivo studies. *Canadian Journal of Physiology and Pharmacology* 75(6):622-628, 1997.

Inokuchi JI, Nagamatsu A. Tripeptidyl carboxypeptidase activity of kininase II (angiotensin-converting enzyme). *Biochemica and Biophysica Acta* 662(2): 300-307, 1981.

Iwai N, Matsunaga M, Kita T, Tei M and Kawai C. Detection of low molecular weight kininogen messenger mRNA in human kidney. *Journal of Hypertension* 6(suppl):399-400, 1988.

Jaspard E, Wei L and Alhenc-Gelas F. Differences in the properties and enzymatic specificities of the two active sites of angiotensin I-converting enzyme (kininase II). Studies with bradykinin and other natural peptides. *Journal of Biological Chemistry* 268: 9496-9503, 1993.

Johnson AR, Skidgel RA, Gafford JT and Erdös EG. Enzymes in placental microvilli: angiotensin I-converting enzyme, angiotensinase A, carboxypeptidase, and neutral endopeptidase ("enkephalinase"). *Peptides* 5, 789-796, 1984.

Kaplan AP and Silverberg M. The coagulation-kinin pathway of human plasma. *Blood* 70:1-16, 1987.

Kelly RA and Smith TW. Drugs used in the treatment of heart failure. In "*Heart Disease*" (ed Braunwald E) 5th ed, ch 16 p471-490 W.B. Saunders Company, Philadelphia. 1997.

Kerr MA and Kenny AJ. The purification and specificity of a neutral endopeptidase from rabbit kidney brush border. *Biochemical Journal* 137: 477-488, 1974.

Kitamura N, Kitagawa H, Fukushima D, Takagaki Y, Miyata T and Nakanishi S. Structural organization of the human kininogen gene and a model for its evolution. *Journal of Biological Chemistry* 260:8610-8617, 1985.

Kumamoto K, Stewart TA, Johnson AR and Erdös EG. Prolylcarboxypeptidase (angiotensinase C) in human cultured cells. *Journal of Clinical Investigation* 67: 210-215, 1981.

Lamontagne D, König A, Bassenge E and Busse R. Prostacyclin and nitric oxide contribute to the vasodilator action of acetylcholine and bradykinin in the intact rabbit coronary bed. *Journal of Cardiovascular Pharmacology* 20:652-657, 1992.

Lamontagne D, Nadeau R and Adam A. Effect of enalaprilat on bradykinin and des-Arg^9-bradykinin release following reperfusion of the ischaemic rat heart. *British Journal of Pharmacology* 115(3): 476-478, 1995.

Lebien TW and McCormack RT. The common acute lymphoblastic leukaemia antigen (CD10) – emancipation from a functional enigma. *Blood* 73:625-634, 1989.

Levin Y, Skidgel RA and Erdös EG. Isolation and characterization of the subunits of human plasma carboxypeptidase N (kininase I). *Proceedings of the National Academy of Sciences of the United States of America* 79: 4618-4622, 1982.

Lindpainter K and Ganten D. The cardiac renin-angiotensin system: An appraisal of present experimental evidence. *Circulation Research* 68: 905-921, 1991.

Linz W, Martorana PA and Scholkens BA. Local inhibition of BK degradation in ischemic hearts. *Journal of Cardiovascular Pharmacology* 15 Suppl 6; S99-S109, 1990.

Linz W and Schölkens BA. In : Bönner G, Schölkens BA and Scicli AG, editors. The role of bradykinin in the cardiovascular action of the converting-enzyme inhibitor ramipril. Chichester : Media Medica, p85-90, 1992.

Linz W, Wiemer G, Gohlke P, Unger T and Scholkens BA. Contrinution of kinins to the cardiovascular actions of angiotensin-converting enzyme inhibitors. *Pharmacological Reviews* 47(1):25-49, 1995.

Llorens-Cortes C, Huang H, Vicart P, Gasc J-M, Paulin D and Corvol P. Identification and characterization of neutral endopeptidase in endothelial cells from venous or arterial origins. *Journal of Biological Chemistry* 267: 14012-14018, 1992.

Lock R and Hessel EA. Probable reversal of protamine reactions by heparin administration. *Journal of Cardiothoracic Anesthesia* 4(5): 605-608, 1990.

Lowenstein E, Johnston EW, Lappas DG, D'Ambra MN, Schneider RC, Dagget WM, Akins CW and Philbin DM. Catastrophic pulmonary vasoconstriction associated with protamin reversal of heparin. *Anesthesiology* 59: 470-473, 1983.

Macdonald RJ, Margolius HS and Erdös EG. Molecular biology of tissue kallikrein. *Biochemical Journal* 253 :313-321, 1988.

Madeddu P, Varoni MV, Palomba D, Emmanueli C, Demontis MP, Glorioso N, Dessi-Fulgheri P, Sarzani R and Anania V. Cardiovascular phenotype of a mouse strain with disruption of bradykinin B2-receptor gene. *Circulation* 96(10): 3570-3578, 1997.

Mandle R Jr, Colman RW and Caplan AP. Identification of pre-kallikrein and HMW-kininogen as a circulating complex in human plasma. *Proceedings of the National Academy of Sciences* 73:4179-4183, 1976.

Mann EA and Lingrel JB. Developmental and tissue-specific expression of rat T-kininogen. *Biochemical and Biophysical Research Communications* 174:417-423, 1991.

Marceau F and Regoli D. Kinin receptors of B_1 type and their antagonists. In "*Bradykinin Antagonists, Basic and Clinical Aspects*". Burch RM, Ed. Marcel Dekker, New York, NY, 1991. P33-49.

Marceau F. Kinin B_1 receptor induction and inflammation. In "*The Kinin System*". Farmer SG, Ed. Academic Press, San Diego, CA, 1997. P144-156.

Marceau F, Hess JF and Bacharov DR. The B_1 receptors for kinins. *Pharmacological Reviews* 50(3):357-386, 1998.

Marks N, Stern F, Chi LM and Berg MJ. Diversity of rat brain cysteine proteinase inhibitors: isolation of low-molecular-weight cystains and higher-molecular-weight T-kininogen-like glycoprotein. *Archives of Biochemistry and Biophysics* 267:448-458, 1988.

Martorana PA, Kettenbach B, Breipohl G, Linz W and Schölkens BA. Reduction of infarct size by local angiotensin-converting enzyme inhibition is abolished by breadykinin antagonist. *European Journal of Pharmacology* 182(2): 395-6, 1990.

Matthews KP, Pan PM, Gardner NJ and Hugli TE. Familial carboxypeptidase N deficiency. *Annals of Internal Medicine* 93: 443-445, 1980.

Matthews KP, Curd JG and Hugli TE. Decreased synthesis of serum carboxypeptidase N (SCPN) in familial SCPN N deficiency. *Journal of Clinical Immunology* 6: 87-91, 1986.

McDonald KM, Mock J, D'Aloia A, Parrish T, Hauer K, Francis G, Stillman A and Cohn JN. Bradykinin antagonism inhibits the antigrowth effect of converting-enzyme inhibition in the dog myocardium after discrete transmural myocardial necrosis. *Circulation* 91: 2043-2048, 1995.

Mombouli JV, Bissiriou I, Agboton V and Vanhoutte PM. Endothelium-derived hyperpolarizing factor: a key mediator of vasodilator action of bradykinin. *Immunopharmacology* 33: 46-50, 1996.

Mombouli JV. ACE Inhibition, Endothelial function and coronary artery lesions – role of kinins and nitric oxide. *Drugs* 54(Suppl5): 12-22, 1997.

Morel DR, Zapol WM, Thomas SJ, Kitain EM, Robinson DR, Moss J, Chenoweth DE and Lowenstein E. C5a and thromboxane generation associated with pulmonary vaso- and broncoconstriction during protamine reversal of heparin. *Anesthesiology* 66: 597-604, 1987.

Müller-Esterl W, Iwanga S and Nakanishi S. Kininogens revisited. *Trends in Biochemical Sciences* 11:336-339, 1986.

Nolly H, Saed G, Scicli AG, et al. The kallikrein-kinin system in cardiac tissue. *Agents and Actions Supplement* 38(II):62-72, 1992.

Nolly H, Carbini LA, Scicli G, Carretero O and Scicli AG. A local kallikrein-kinin system is present in rat hearts. *Hypertension* 23(2):919-923, 1994.

O'Connell JE, Jardine AG, Davies DL, et al. Renal and hormonal effects of chronic inhibition of neutral endopeptidase (EC 3.4.24.11) in normal man. *Clinical Science* 13: 797-804, 1993.

Opie LH. The Renin-angiotensin aldosterone system and other proposed sites of action of angiotensin-converting enzyme inhibitors. In "*Angiotensin-Converting Enzyme Inhibitors: Scientific Basis for Clinical Use 2nd ed*". Opie LH ed. Wiley-Liss Authors' Pulbishing House, New York, NY. 1994 pp1-22.

Opie LH, Poole-Wilson PA, Sonnenblick E and Chatterjee K. Angiotensin-Converting Enzyme Inhibitors. Contrasts with Conventional Vasodilators. In "*Drugs for the Heart 4th ed.*". Opie LH ed. WB Saunders Company, Philadelphia, PA. 1995, pp105-144.

Orawski AT and Simmons WH. Purification and properties of membrane-bound aminopeptidase P from rat lung. *Biochemistry* 34(35): 11227-11236, 1995.

Parmley WW. Evolution of angiotensin-converting enzyme inhibition in hypertension, heart failure, and vascular protection. *American Journal of Medicine* 105(1A): 27S-31S, 1998.

Pham I, Gonzales W, Doucet J, Fournié-Zaluski MC, Roques BP and Michel JB. Effects of angiotensin-converting enzyme and neutral endopeptidase inhibitors: influence of bradykinin. *European Journal of Pharmacology* 296:267-276, 1996.

Pham I, Gonzales W, El-Amrani AI, Fournié-Zaluski MC, Philippe M, Laboulandine I, Roques B and Michel JB. Effects of converting-enzyme inhibitor and neutral endopeptidase inhibitor on blood pressure and renal function in experimental hypertension. *Journal of Pharmacology and Experimental Therapeutics* 265 :1339-1347, 1993.

Pham I, El Amrani EI, Fournié-Zaluski MC, Corvol P, Roques BP and Michel JB. Effects of the selective neutral endopeptidase inhibitor, retrothiorphan, on renal function and blood pressure in normotensive and DOCA-salt hypertensive rats. *Journal of Cardiovascular Pharmacology* 20: 847-855, 1992.

Piedimonte G, Nadel JA, Long CS and Hoffman JIE. Neutral endopeptidase in the heart. Neutral endopeptidase inhibition prevents isoproterenol-induced hypoperfusion in rats by reducing bradykinin degradation. *Circulation Research* 75: 770-779, 1994.

Plummer TH and Hurwitz MY. Human plasma carboxypeptidase N. Isolation and characterization. *Journal of Biological Chemistry* 259: 3053-3057, 1978.

Prechel MM, Orawski AT, Maggiora LL and Simmons WH. Effect of a new aminopeptidase P inhibitor, apstatin, on bradykinin degradation in the rat lung. *Journal of Pharmacology and Experimental Therapeutics* 275: 1136-1142, 1995.

Raymond P, Bouvier M, Drapeau G, Morais R and Adam A. Bradykinin decreases T-kininogen synthesis in a rat hepatoma cell line: evidence of bradykinin B_2–type receptors. *Peptides* 17(7):1171-1176, 1996.

Regoli D and Barabé J. Pharmacology of bradykinin and related kinins. *Pharmacological Reviews* 32(1): 1-46, 1980.

Rehli M, Krause SW, Kreutz M and Andreesen R. Carboxypeptidase M is identical to the MAX.1 antigen and its expression is associated with monocyte to macrophage differentiation. *Journal of Biological Chemistry* 270: 15644-15649, 1995.

Richards AM, Wittert GA, Espiner EA, Yandle TG, Ikram H and Frampton C. Effect of inhibition of endopeptidase 24.11 on response to angiotensin II in human volunteers. *Circulation Research* 71: 1501-1507, 1992.

Roques BP, Noble F, Crine P and Fournié-Zaluski MC. Inhibitors of neprilysin: design, pharmacological and clinical applications. *Methods in Enzymology* 248 : 263-282, 1995.

Ronco P, Pollard H, Galceran M, Delauche M, Schwartz JC and Verroust P. Distribution of enkephalinase (membrane metalloendopeptidase, E.C. 3.4.24.11) in rat organs. Detection using a monoclonal antibody. *Laboratory Investigation* 58: 210-217, 1988.

Ruskoaho H. Atrial natriuretic peptide: synthesis, release and metabolism. *Pharmacological Reviews* 44: 479-602, 1992.

Ryan JW, Berryer P, Chung AY and Sheffy DH. Characterization of rat pulmonary vascular aminopeptidase P in vivo: role in the inactivation of bradykinin. *Journal of Pharmacology and Experimental Therapeutics* 269: 941-947, 1994.

Salvino JM, Seoane PR, Douty B, Awad MM, Dolle RE, Houck WT, Faunce DM and Sawutz DG. Design of non peptide competitive antagonists of the bradykinin B2 receptor. *Journal of Medicinal Chemistry* 36:2583-2584, 1993.

Sato F and Nagasawa S. Mechanism of kinin release from human low-molecular-mass-kininogen by the synergistic action of human plasma kallikrein and leucocyte elastase. *Biological Chemistry* 369:1009-1017, 1988.

Schapira M, Scott CF and Colman RW. Protection of human plasma kallikrein from inactivation by C1 inhibitor and other protease inhibitors. The role of high molecular weight kininogen. *Biochemistry* 20:2738-2743, 1981.

Schmaier AH, Kuo A, Lundberg D, Murray S and Cines DB. The expression of high molecular weight kininogen on human umbilical vein endothelial cells. *Journal of Biological Chemistry* 263: 16327-16333, 1988.

Schwartz JC, Costentin J and Lecomte JM. Pharmacology of enkephalinase inhibitors. *Trends in Pharmacological Sciences* 6: 472-485, 1985.

Shimada Y and Avkiran M. Attenuation of reperfusion arrhythmias by selective inhibition of angiotensin-converting enzyme kininase II in the ischemic zone – mediated by endogenous bradykinin. *Journal of Cardiovascular Pharmacology* 91: 428-438, 1996.

Sidorowicz W, Canizaro PC and Behal FJ. Kinin cleavage by human erythrocytes. *American Journal of Hematology* 17: 383-391, 1984.

Sidorowicz W, Szechinski J, Canizaro PC and Behal FJ. Cleavage of the Arg^1-Pro^2 bond of bradykinin by a human lung peptidase: isolation, characterization and inhibition by several β-lactam antibiotics. *Proceedings of the Society for Experimental Biology and Medicine* 175: 503-509, 1984.

Simmons WH and Orowski AT. Membrane-bound aminopeptidase P from bovine lung. Its purification, properties and degradation of bradykinin. *Journal of Biological Chemistry* 267: 4897-4903, 1992.

Skidgel RA, Wickstrom E, Kumamoto K and Erdös EG. Rapid radioassay for prolylcarboxypeptidase (angiotensinase C). *Anals of Biochemistry* 118(1): 113-119, 1981.

Skidgel RA, Davis RM and Tan F. Human carboxypeptidase M: purification and characterization of a membrane-bound carboxypeptidase that cleaves peptide hormones. *Journal of Biological Chemistry* 264(4): 2236-2241, 1989.

Skidgel RA and Erdös EG. Biochemistry of Angiotensin converting enzyme. In "*The Renin Angiotensin System*", Vol.1 (eds. JIS Roberts and MG Nicholls), 1993, pp 10.1.-10.10. Gower Medical Publishers, London.

Skidgel RA, McGwire GB and Li X-Y. Membrane anchoring and release of carboxypeptidase M: Implications for extra-cellular hydrolysis of peptide hormones. *Immunopharmacology* 32: 48-52, 1996.

Sterchi EE, Naim HY, Lentze MJ, Hauri Hp and Fransen JA. N-benzoyl-l-tyrosyl-p-aminobenzoic acid hydrolase: a metalloendopeptidase of the human intestinal microvillus membrane which degrades biologically active peptides. *Archives of Biochemistry and Biophysics* 265: 105-118, 1988.

Sterchi EE, Hahn D and Lottaz D. Endopeptidase-24.18. In "*Cell-Surface Peptidases in Health and Disease*" (eds. Kenny AJ and Boustead CM), 1997, pp 155-174. Bios Scientific Publishers, Oxford.

Takano M, Yokoyama K, Yayama K and Okamoto H. Rat fibroblasts synthesize T-kininogen in response to cyclic-AMP, prostaglandin E2 and cytokines. *Biochemical and Biophysical Acta* 1268:107-114, 1995.

Tan F, Morris PW, Skidgel RA and Erdös EG. Sequencing and cloning of human prolylcarboxypeptidase (angiotensinase C): similarity to both serine carboxypeptidase and prolylendopeptidase families. *Journal of Biological Chemistry* 268: 16631-16638, 1993.

Tisljar U. Thimet oligopeptidase – a review of a thiol-dependent metalloendopeptidase also known as Pz-peptidase, endopeptidase 24.15 and endo-oligopeptidase. *Biological Chemistry*, Hoppe-Seyler 374: 91-100, 1993.

Trippodo NC, Panchal BC and Fox M. Repression of Angiotensin II and potentiation of Bradykinin contribute to the synergistic effects of dual metalloprotease inhibition in heart failure. *Journal of Pharmacology and Experimental Therapeutics* 272:619-627, 1995a.

Trippodo NC, Robl JA, Asaad MM, Bird JE, Panchal BC, Schaeffer TR, Fox M, Giancarli MR and Cheung HS. Cardiovascular effects of the novel dual inhibitor of neutral endopeptidase and angiotensin-converting enzyme BMS-182657 in experimental

hypertension and heart failure. *Journal of Pharmacology and Experimental Therapeutics* 275: 745-752, 1995b.

Trippodo NC, Robl JA, Asaad MM, Fox M, Panchal BC and Schaeffer TR. Effects of omapatrilat in low, normal and high renin experimental hypertension. *American Journal of Hypertension* 11:363-372, 1998.

Turner AJ. Endopeptidase 24.11 and neuropeptide metabolism. In "*Neuropeptides and Their Peptidases*" (ed. Turner AJ), pp 183-201. Ellis-Horwood, Chichester, 1987.

Turner AJ. Neuropeptide inactivation in the central nervous system. In "*Cell-Surface Peptidases in Health and Disease*" (eds. Kenny AJ and Boustead CM), 1997, pp 275-301. Bios Scientific Publishers, Oxford.

Turner AJ and Hooper NM. Three glycolipid-anchored peptidases: membrane dipeptidase, aminopeptidase P and carboxypeptidase M. In "*Cell-Surface Peptidases in Health and Disease*" (eds. Kenny AJ and Boustead CM), 1997, pp 219-238. Bios Scientific Publishers, Oxford.

Ueda S, Elliot HL, Morton JJ and Connell JMC. Enhanced pressor response to angiotensin I in normotensive men with the deletion genotype DD for angiotensin-converting enzyme. *Hypertension* 25:1266-1269, 1995.

Ura N, Carretero OA and Erdös EG. Role of renal endopeptidase 24.11 in kinin metabolism *in vitro* and *in vivo*. *Kidney International* 32 : 507-513, 1987.

Vanhoof G, De Meester I, Goossens F, Hendriks D, Scharpe S and Yaron A. Kininase activity in human platelets: cleavage of the Arg1-Pro2 bond of bradykinin by human aminopeptidase P. *Biochemical Pharmacology* 44: 479-487, 1992.

Van Iwaarden AF, de Groot PG and Bouma BN. The binding of high molecular weight kininogen to cultured human endothelial cells. *Journal of Biological Chemistry* 263: 4698-4703, 1989.

Vavrek R and Stewart JM. Competitive antagonists of bradykinin. *Peptides* 6:161-164, 1985.

Vegh A, Papp JG and Parrat JR. Attenuation of the antiarrhythmic effects of ischemic preconditioning by blockade of bradykinin B2 receptors. *British Journal of Pharmacology* 113: 1167-1172, 1994.

Vijayaraghavan J, Scicli AG, Carretero OA, Slaughter C, Moomaw C and Hersh LB. The hydrolysis of endothelins by neutral endopeptidase 24.11 (enkephalinase). *Journal of Biological Chemistry* 265: 14150-14155, 1990.

Waeber B, Aubert JF, Vavrek R, Stewart JM, Nussberger J and Brunner HR. Role of bradykinin in the blood pressure response to acute angiotensin converting enzyme inhibition in rats. *Journal of Hypertension* 4(Suppl6): S597-S598, 1986.

Ward PE. Metabolism of bradykinin and bradykinin analogs. In "*Bradykinin Antagonists, Basic and Clinical Research*". Burch RM ed. New York: Marcel Dekker Company, pp147-170, 1991.

Ward PE, Russel JS and Vaghy PL. Angiotensin and bradykinin metabolism by peptidases identified in skeletal muscle. *Peptides* 16: 1073-1078, 1995.

Weare JA. Activation/inactivation of human angiotensin I converting enzyme following chemical modifications of amino groups near the active site. *Biochemical and Biophysical Research Communications* 104: 1319-1326, 1982.

Welches WR, Brosnihan KB and Ferrario CM. A comparison of the properties and enzymatic activities of three angiotensin processing enzymes: angiotensin converting enzyme, prolyl endopeptidase and neutral endopeptidase 24.11. *Life Sciences* 52(18): 1461-1480, 1993.

Whalley ET, Loy SD, Modifieri D, Blodgett JK and Cheronis JC. A novel potent bis(succinimido)-hexane peptide heterodimer antagonist (CP-0364) of bradykinin B_1 and B_2 receptors: *in vitro* and *in vivo* studies. *British Journal of Pharmacology* 107: 257P, 1992.

Wilk S. Minireview: prolyl endopeptidase. *Life Sciences* 33: 2149-2157, 1983.

Wirth K, Hock FJ, Albus U, Linz W, Alpermann HG, Anagnostopoulos H, Henke St, Breipohl G, König W and Knolle J. Hoe 140 a new potent and long acting bradykinin-antagonist: *in vivo* studies. *British Journal of Pharmacology* 102:774-777, 1991.

Wolz RL and Bond JS. Meprins A and B. *Methods in Enzymolgy* 248:325-345, 1995.

Yamada H, Fabris B, Allen AM, Jackson B, Johnston CI and Mendelsohn FAO. Localization of angiotensin converting enzyme in rat heart. *Circulation Research* 68(1): 141- 149.

Yayama K, Nagaoka N, Takano M and Okamoto H. Kininogen expression by the rat cardiac myocytes. The Kallikrein-Kinin System from molecular biology to pathophysiology. The Fifteenth International Conference on Kinins, October 19th to 24th, 1998. Abstract P-I-2, 1998.

Zak R. Cardiac hypertrophy and atrophy. In *"The Heart – Physiology, Metabolism, Pharmacology and Therapy"* Opie LH ed. Grune and Stratton Ltd., London. 1984, pp 198-209.