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# PKB mediates Insulin-Like Growth Factor 1-induced phosphorylation and nuclear export of Histone Deacetylase 5 via NADPH Oxidase 4 activation in vascular smooth muscle cells

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# PKB mediates Insulin-Like Growth Factor 1-induced phosphorylation and nuclear export of Histone Deacetylase 5 via NADPH Oxidase 4 activation in vascular smooth muscle cells

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# Résumé

L'Insulin-like growth factor-1 (IGF-1) est un peptide vasoconstricteur qui joue un rôle proéminent dans la progression des maladies cardiovasculaires, grâce à la génération d'espèces réactives de l'oxygène (*ERO*), ainsi que par l'hyperactivation des voies de signalisation qui promeuvent la croissance et l'expression aberrante des gènes. Les histones désacétylases (HDACs), par leur aptitude à modifier le statut d'acétylation des résidus lysine dans les protéines d'histones et non-histones, régulent la transcription des gènes. Des études récentes ont montré qu'une activation accrue des HDACs, notamment HDAC5, est associée à des troubles vasculaires tels que l'athérosclérose. Cependant, le rôle de l'IGF-1 dans l'activation des HDACs par phosphorylation demeure mal caractérisé. Donc, dans les études présentes, on a examiné l'effet de l'IGF-1 sur la phosphorylation de HDAC5 dans les cellules musculaires lisses vasculaires (CMLV) de type A10 et on a identifié les voies de signalisation impliquées dans ce processus. Le traitement des CMLV avec l'IGF-1 a augmenté la phosphorylation de HDAC5 sur la serine 498 de manière temps- et dose-dépendante. La pré-incubation des cellules avec l'AG1024, un inhibiteur pharmacologique du récepteur membranaire à l'IGF-1, a significativement atténué la phosphorylation d'HDAC5 induite par l'IGF-1. Par contre, le prétraitement avec l'AG1478, un inhibiteur du récepteur du facteur de croissance épidermique, n'a pas eu d'effet significatif sur les niveaux de phosphorylation d'HDAC5. En outre, le blocage pharmacologique de la voie MAP kinase avec divers inhibiteurs (PD98059, UO126, SP600125 et SB203580) n'a pas abrogé la phosphorylation d'HDAC5, cependant les inhibiteurs de la voie protéine kinase B (PKB)/PI3-K, SC-66 et wortmannine respectivement, ont complètement aboli la phosphorylation d'HDAC5 induite par l'IGF-1. Ces données ont été confirmées par des expériences d'immunofluorescence et de silençage génique de PKB par interférence d'ARN. En outre, le prétraitement des cellules avec le diphénylèneiodonium (DPI) et l'apocynine, deux inhibiteurs de la NAD(P)H oxydase, ainsi que l'antioxydant N-acétyl-cystéine (NAC), a atténué la phosphorylation de HDAC5 et de PKB par l'IGF-1. De plus, le silençage génique de Nox4, la principale NAD(P)H oxydase des CMLV, a atténué la phosphorylation d'HDAC5 induite par l'IGF-1. De plus, l'utilisation des techniques d'extraction nucléaire a indiqué que le SC-66 et le DPI inhibent l'exportation nucléaire de HDAC5 induit par IGF-1. En résumé, ces données suggèrent que l'IGF-1 induit la phosphorylation et l'exportation nucléaire de HDAC5 de façon ERO et PKB-dépendante dans les CMLV.

**Mots-clés**: IGF-1, HDAC5, PKB, PI3-K, MAPK, ROS, VSMC

# Abstract

Insulin-like growth factor 1 (IGF-1), a potent mitogenic and vasoactive factor, has been shown to play a role in the development of cardiovascular diseases. This occurs through the generation of reactive oxygen species (ROS) as well as through the hyperactivation of mitogenic and growth promoting signaling pathways and the subsequent alteration in gene expression. Histone deacetylases (HDACs), by their ability to modify the acetylation status of the lysine residues in histone and non-histone proteins, regulate gene transcription. Recent studies have demonstrated that a heightened activation of HDACs, notably HDAC5, is associated with vascular disorders such as atherosclerosis. However, a role of IGF-1 in HDAC phosphorylation and activation has not been investigated. Therefore, in the present studies, we examined the effect of IGF-1 on the phosphorylation of HDAC5 in vascular smooth muscle cells (VSMCs) and identified the signaling pathways involved in this process. Treatment of A10 VSMCs with IGF-1 enhanced the phosphorylation of HDAC5 at serine 498 in a time and dose-dependent fashion. Pretreatment of cells with AG1024, a selective pharmacological inhibitor of IGF-1R, significantly inhibited IGF-1-induced HDAC5 phosphorylation in A10 VSMCs whereas AG1478, a selective inhibitor of epidermal growth factor receptor (EGFR), did not have an inhibitory effect on the levels of phospho-HDAC5. Pharmacological blockade of the MAPK pathway with PD98059, UO126, SP600125 and SB203580 had no effect on HDAC5 phosphorylation, whereas inhibitors of the PI3K/ PKB pathways, wortmannin and SC-66 respectively, almost completely attenuated IGF-1-induced HDAC5 phosphorylation. These findings were confirmed by immunofluorescence localization of phospho-HDAC5 and by siRNA-induced silencing of PKB. In addition, pretreatment of A10 VSMCs with Diphenyleneiodonium (DPI) and apocynin, two NAD(P)H oxidase inhibitors, as well as the antioxidant N-Acetyl-Cysteine (NAC), resulted in an attenuation of IGF-1-induced HDAC5 and PKB phosphorylation. Furthermore, siRNA-induced silencing of Nox4, the main NADPH oxidase expressed in VSMC, inhibited IGF-1 induced HDAC5 phosphorylation. Moreover, IGF-1-induced phosphorylation of HDAC5 resulted in its nuclear export, which was reversed by blockade of PKB by SC-66 or NAD(P)H oxidase inhibition by DPI. In summary, these data demonstrate that IGF-1 induces the phosphorylation and nuclear export of HDAC5 in a Nox4-derived ROS- and PKB-dependent fashion in VSMCs.

**Keywords:** IGF-1, HDAC5, PKB, PI3-K, MAPK, ROS, VSMC

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# List of abbreviations and acronyms

| | |
|---|---|
| ALS | acid labile subunit |
| AMPK | AMP-activated protein kinase |
| AngII | angiotensin II |
| ATF2 | activating transcription factor 2 |
| Ca2+ | calcium |
| CaMK | Ca2+/calmodulin-dependent protein kinase |
| cAMP | cyclic adenosine monophosphate |
| CVD | cardiovascular disease |
| EC | endothelial cell |
| EGFR | epidermal growth factor receptor |
| eNOS | endothelial nitric oxide synthase |
| ER | endoplasmic reticulum |
| ERK1/2 | extracellular signal-regulated kinase ½ |
| ET-1 | endothelin 1 |
| FOXO | forkhead box O protein |
| GDP | guanosine diphosphate |
| GH | growth hormone |
| GPCR | G-protein coupled receptor |
| Grb-2 | growth factor receptor-binding protein 2 |
| GSK-3 | glycogen synthase kinase 3 |
| GTP | guanosine-5'-triphosphate |
| HAT | histone acetyltransferase |
| HDAC | histone deacetylases |
| HSP | heat shock protein |
| IGF-1/IGF-2 | insulin-like growth factor 1/ insulin-like growth factor 2 |
| IGF-1R/IGF-2R | insulin-like growth factor type 1 receptor/ insulin-like growth factor type 2 receptor |

| | |
|---|---|
| IGFBP 1-6 | insulin-like growth factor binding proteins 1-6 |
| IR | insulin receptor |
| IRS | insulin receptor substrate |
| JNK 1/2/3 | c-Jun N-terminal kinase 1/2/3 |
| JAK/STAT | Janus kinase/signal transducer and activator of transcription |
| KLF4 | Kruppel-like factor 4 |
| LDL | low-density lipoprotein |
| MAPK | mitogen activated protein kinase |
| Mef2 | myocyte enhancer factor-2 |
| MEK | mitogen extracellular regulated kinase |
| MMP | matrix metalloproteinase |
| mTOR | mammalian target of rapamycin |
| NAD+ | nicotinamide adenine dinucleotide |
| Nox | NADPH oxidase |
| NO | nitric oxide |
| PDGF | platelet-derived growth factor |
| PDGFR | platelet-derived growth factor receptor |
| PDK1/2 | phosphoinositide-dependent kinase 1 and 2 |
| PH domain | pleckstrin homology domain |
| PI3-K | phosphatidylinositol 3-kinase |
| PIP2 | phosphatidylinositol 4,5-bisphosphate |
| PIP3 | phosphatidylinositol (3,4,5) – triphosphate |
| PKA | protein kinase A |
| PKB | protein kinase B |
| PKC | protein kinase C |
| PKD | protein kinase D |
| PTK | protein tyrosine kinase |
| R-PTK | receptor protein tyrosine kinase |
| ROS | reactive oxygen species |
| SERCA | sarco/endoplasmic reticulum Ca2+-ATPase |

| | |
|---|---|
| SH2 | src homology 2 |
| SIRT | sirtuin |
| SOS | son of sevenless |
| SRF | serum response factor |
| STIM1 | stromal interaction molecule 1 |
| T3 | triiodothyronine |
| TF | transcription factor |
| UTR | untranslated region |
| VSMC | vascular smooth muscle cell |

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# Chapter 1

# Introduction

According to the World Health Organization (WHO), cardiovascular diseases (CVDs) are the leading causes of death worldwide, with an estimated 17.5 million CVD-related deaths in 2012 (1). CVDs are associated with vascular remodeling characterized by increased vascular resistance (2). Abnormal migration, growth and proliferation of vascular smooth muscle cells (VSMCs) have been implicated in vascular remodeling and vascular diseases such as atherosclerosis, hypertension, intimal hyperplasia and restenosis (3, 4, 5). Vasoactive peptides and growth factors, such as insulin-like growth factor 1 (IGF-1), have been suggested to play a key role in vascular remodeling by promoting VSMC proliferation, migration and hypertrophy (6, 7). Studies have suggested that IGF-1, a potent mitogenic and vasoactive factor (8), contributes to the aberrant phenotype of VSMCs through the hyperactivation of growth promoting signaling pathways and the subsequent alteration in gene expression (9). Histone deacetylases (HDACs), by their ability to modify the acetylation status of lysine residues in histone and non-histone proteins, have been suggested to be involved in epigenetic regulation of gene transcription (10) and recent studies have suggested that HDACs may be involved in vascular remodelling events leading to CVDs via their effects on genes implicated in cell growth, proliferation and migration (11). Epigenetic modifications, such as acetylation, have emerged as important mechanisms that modulate gene expression without changing the DNA sequence and thus provide rapid and reversible regulation of many genes (12). The following sections provide a brief overview on the structure and function of IGFs and HDACs, as well IGF-1's mechanism of action, with particular interest to its role in the cardiovascular system.

# 1. General properties of IGFs

Insulin-like growth factors (IGFs) belong to a family of polypeptide hormones, also called somatomedins (13). Prior to this nomenclature, IGFs were first termed sulfation factors because of their ability to stimulate the sulfation of proteoglycans that are present in cartilage (14). To date, the IGF system consists of IGF-1 and IGF-2, two cell surface receptors (IGF1R and IGF2R) and IGFBP-1 to IGFBP-6, six high affinity IGF-binding proteins, as well as IGFBP degrading enzymes. The IGF system has been shown to be involved in the regulation of important cellular and physiological processes, including development, growth, cell regulation and metabolism (15).

## 1.1 Structural features of IGFs

As previously mentioned, there exist two IGFs, IGF-1 and IGF-2, and their structural features can be examined at the gene as well as the protein level.

### 1.1A Structural features of IGF-1

The IGF-1 gene is located on human chromosome 12 (16) and the gene sequence contains six exons and five introns spanning 80kb of chromosomal DNA (17). Exons 3 and 4 encode a portion of the N-terminal signal sequence, the complete sequence of the mature protein and a portion of the C terminus. Exons 1, 2, 5 or 6 can be alternatively spliced leading to four splice variants or mRNA subtypes containing differences in the 5' and 3' untranslated regions (UTR) (17). The IGF-1 gene sequence has been shown to be highly conserved amongst species (18). Two promoter regions in the IGF-1 gene sequence have been identified to date and are

termed P1 and P2. P1 and P2 are located upstream of exon 1 and exon 2, respectively, and do not contain TATA and CAAT elements (19).

The IGF-1 protein is a single chain polypeptide composed of 70 amino acid residues having a molecular weight of 7.6 kDa (20). The IGF-1 sequence is known for a number of different species and is shown to be highly conserved (18). It has a 70% sequence homology with IGF-2 and a 50% sequence homology with proinsulin. IGF-1 protein is comprised of 4 domains, with domain A and B having the most homology with insulin (60 to 70% similarity) (20). The A domain, which is made up of 21 residues, contains alpha helix 2 and alpha helix 3 joined by a loop. IGF-1's B domain consists of 29 residues and contains an extended N-terminal coil followed by a tight turn and a central alpha helix 1, forming a hydrophobic core (20, 21). The C and D domains, 12 and 8 amino acids respectively, have no homology with proinsulin and form the carboxy terminus of the protein (22). The three dimensional structure of IGF-1, through nuclear magnetic resonance (NMR) and X-ray crystallography methods, has been reported by several groups (20, 22). Figure 1 depicts the IGF-1 mRNA transcript and protein structure.

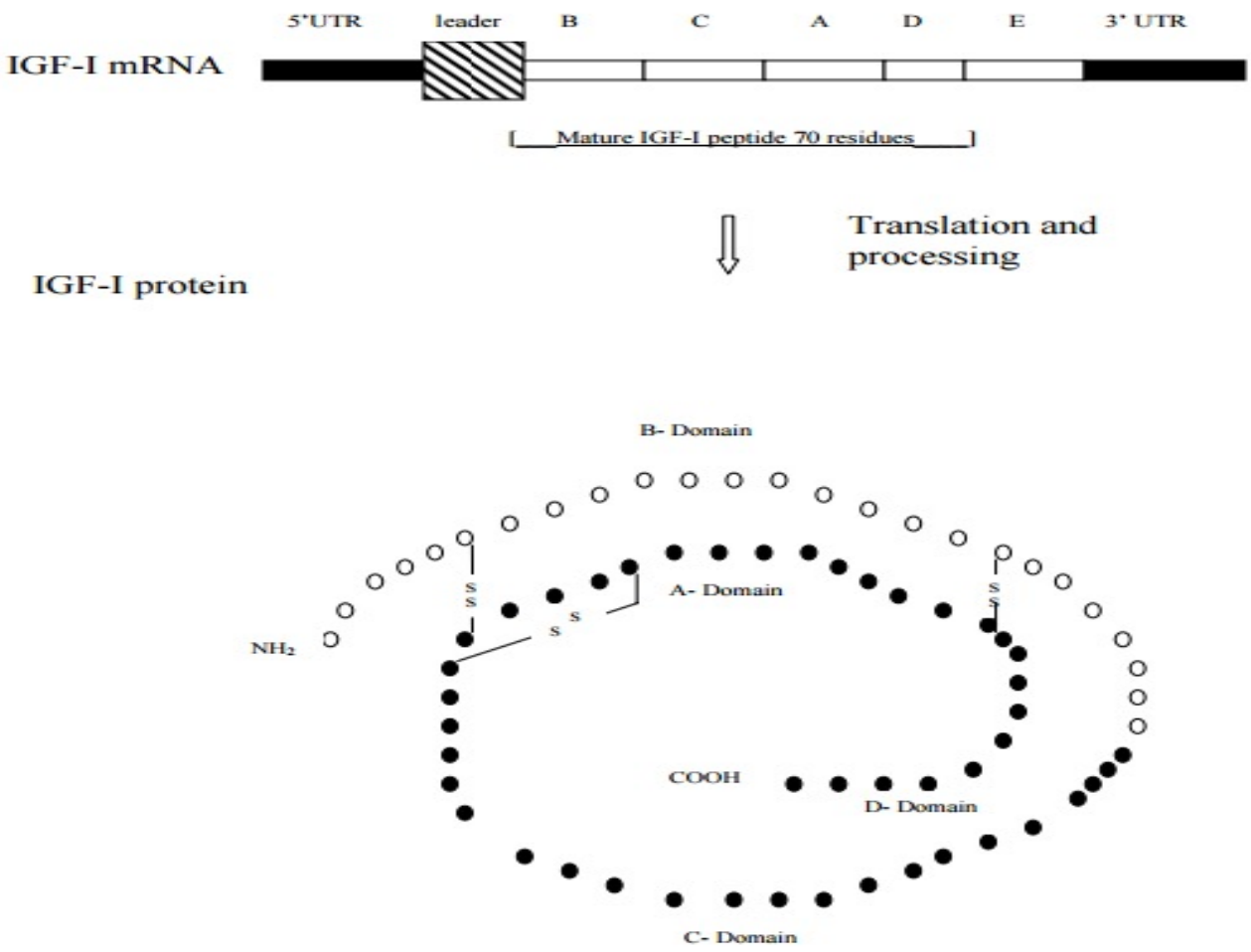


**Figure 1: Schematic illustration of the IGF-1 mRNA transcript and protein structure. The mRNA transcript possesses a 5'UTR and leader sequence derived primarily from exons 1 or 2 depending on the mRNA subtype. A 3'UTR is derived from exons 5 and 6 and is also dependent on mRNA subtype. Exons 3 and 4 encode B, C, A, D and E domains which are translated into pre-protein. Proteolytic cleavage of the C-terminal E domain results in the formation of a mature protein. The protein contains three disulfide bridges (17, 20-22).**

### 1.1B Structural features of IGF-2

The IGF-2 gene is found on chromosome 11. IGF-2 preprohormone consists of 180 amino acids and contains a carboxy-terminal peptide of 89 amino acids and a signal peptide of 24 amino acids, both of which are cleaved following translation to yield a 67-amino acid plasma protein (23).

IGF-2 is a single chain polypeptide hormone composed of 67 amino acids with a molecular weight of 7.4kDa (22). Like IGF-1, IGF-2 contains A, B, C and D domains. The A domain is comprised of 21 residues, an alpha helix 2 (Glu44-Phe48) and an alpha helix 3 (Ala54-Tyr59). Its B domain has 29 residues and a central alpha helix 1 (Gly10-Val20). IGF-2's C and D domains have the same properties as IGF-1's C and D domains (21, 22).

## 1.2 Regulation of IGF-1 levels

During prenatal and postnatal growth and development, serum IGF-1 levels vary from 20ng/mL in fetal stages to 200 ng/mL after birth (24). IGF-1 concentration can be regulated in a number of ways at different functional levels. Namely, at the level of IGF-1 synthesis, at the post-translational stage, and by interactions with insulin-like growth factor-binding proteins (IGFBPs).

### 1.2A Regulation of IGF-1 at the level of synthesis

IGF-1 is predominantly synthesized in the liver and its plasma concentration is regulated by liver growth hormone (GH) depending on nutritional status. Plasma IGF-1 levels are maintained at a normal level through a negative-feedback mechanism that suppresses GH synthesis in the pituitary gland when IGF-1 levels are high (25). In addition to synthesis in the liver, IGF-1 can also be synthesized in many peripheral tissues, including skeletal muscle, bone, and cartilage, enabling it to act locally as an autocrine/paracrine factor or more distantly as an endocrine factor (26). Furthermore, IGF-1 has also been shown to be synthesized and secreted by vascular cells. Studies indicate the existence of three IGF-1 mRNA transcripts localized in the smooth muscle layer of the adult rat aorta and two IGF-1 mRNA transcripts in aortic endothelial cells (27).

Three IGF-1 transcription factors (TFs) have been identified, CAAT-box/enhancer binding protein (C/EBP), hepatocyte nuclear factor-1 (HNF-1) and HNF-3, and they bind to six binding sites within the P1 (expressed in all tissues) and P2 (expressed primarily in the liver) promoter regions (28). Once transcription is initiated at these promoter regions, a pre-pro-IGF-1 precursor protein is formed, which contains a signal peptide, a signal cleavage site, the mature IGF-1, a pro-protein convertase cleavage site and a C-terminal E-peptide extension (29). During translation, which occurs in the endoplasmic reticulum (ER), the N-terminal signal sequence is cleaved and the pro-IGF-1 polypeptide (mature IGF-1 and E-peptide) is formed.

A number of factors have been found to regulate the bioactive levels of IGF-1. For example, insulin has been shown to increase liver IGF-1 transcription in vivo (30) and in primary hepatocyte cultures (31). GH also has been found to increase IGF-1 mRNA in primary hepatocytes (32) and in the liver (in vivo) (33). Furthermore, glucagon (34) and the thyroid derived hormone, triiodothyronine (T3) (35), have been shown to increase IGF-1 mRNA in primary hepatocytes. In addition to these hormones, genetic factors and age are also important determinants in controlling the variability in IGF-1 levels (25).

**1.2B Regulation of IGF-1 at the post-translational stage**

Prior to secretion, the pro-IGF-1 polypeptide can undergo various post-translational modifications. It can undergo intracellular protease cleavage, which disconnects the mature IGF-1 protein from the E-peptide. Additionally, it can be secreted without cleavage or it may be subjected to E-peptide N-glycosylation and then be secreted. Thus, there are three resulting forms of IGF-1: mature IGF-1, glycosylated-pro-IGF-1 and nonglycosylated-pro-IGF-1 (36).

### 1.2C Regulation of IGF-1 through IGFBP interaction

Following synthesis and release, IGF-1 can be found in a free form (2%) or bound to IGFBPs (98%) (37). IGFBPs, which regulate the level of free IGFs in circulation, are composed of a family of six proteins (IGFBP-1-6) that bind to IGFs with high affinity and specificity and a family of IGFBP-related proteins (IGFBP-rPs), which are structurally similar to the IGFBPs but bind IGFs with much lower affinity (38).

IGFBPs were initially discovered while purifying IGF-1 from serum (39). To date, all six members of the IGFBP family have been cloned and their complete sequences have been elucidated (40). IGFBPs have a highly conserved structure consisting of three domains: an N-terminal and a C terminal domain, which are both cysteine rich, and a highly variable central domain. The N- and C-terminal domains mediate IGF-binding, whereas the central domain differs between all IGFBPs and serves as a hinge between the N- and C-terminal domains (41). Post-translational IGFBP modifications, such as proteolysis, phosphorylation and glycosylation, occur in the central domain and have the ability to alter IGF-binding affinity (42).

IGFBP-3 is the most common IGF binding protein in the blood stream followed by IGFBP-2, which is produced in the liver. Most of the circulating IGF-1 and IGF-2 are associated with a high molecular weight complex (~150 kDa) consisting of IGFBP-3 and the acid labile subunit (ALS) (43). Following separation from the ternary complex, the binary complexes of IGFBP-IGF are removed from the circulation and cross the endothelium to reach their target tissues and to interact with cell surface receptors (43). In addition to transporting IGFs, IGFBPs can protect IGFs from degradation, thus prolonging their half-life. Furthermore, IGFBPs can act

as promoters by promoting IGF interaction with their receptor, as well as inhibitors by sequestering IGF from its receptor, as the IGFBPs have a higher affinity for the IGFs than the receptors (44).

IGFBP action is controlled by IGFBP proteases, which belong to a superfamily of proteases with high-affinity for IGFBPs. These proteases are key modulators of the levels of IGFBPs and ultimately regulate the bioactivity and downstream actions of IGFs (45). Matrix metalloproteinases (MMPs), which degrade several extracellular matrix molecules including collagens, elastins and proteoglycans, are also known to be active against IGFBPs (46).

## 2. Functional features of IGF-1

To carry out their biological actions, including regulation of development, growth and metabolism, IGFs interact with IGF receptors. IGF-1 exerts its physiological actions through its interaction with IGF-1R, a high-affinity receptor specific to IGF-1. IGF-1 has also been shown to bind to IGF-2R with low affinity, though IGF-2 is the high affinity ligand for IGF-2R (21). Additionally, there is high homology between IGF-1R and the insulin receptor and activation of these receptors triggers similar downstream signaling pathways. Insulin and IGF-1 demonstrate high affinity binding with their specific receptors, however insulin can also bind and activate IGF-1R. Therefore, though IGF-1 is the main activator of IGF-1R, insulin may potentially contribute to its activation as well (47).

### 2.1 **IGF-1 receptor**

Human IGF-1R is a ubiquitously expressed product of a single-copy gene located on chromosome 15 (47). In terms of its structure, IGF-1R is a heterotetramer consisting of two extracellular ligand binding α-subunits that are linked by disulfide bonds to each other and to two transmembrane β-subunits that contain intrinsic tyrosine kinase activity, which is believed to be essential for most of the receptor's biological effects (47).

IGF-1R is first synthesized as a single polypeptide chain, known as the IGF-1 preproreceptor, and is then cleaved post-translationally to form a proreceptor. The proreceptor is then glycosylated, folded and dimerized by calnexin and calreticulin (48), two chaperones, and finally it is transported to the Golgi apparatus to yield the mature IGF-1 receptor. Mature IGF-1R bears strong similarities in biochemical structure to the insulin receptor (IR) (49). IGF-1R is composed of three domains: an N-terminal extracellular hormone-binding domain, a transmembrane region, and a C-terminal intracellular domain with intrinsic kinase activity and regulatory residues (50). IGF-1R's α-subunit and 194 residues of the β-subunit are situated in the N-terminal and this region includes 11 potential N-linked glycosylation sites, as well as two homologous leucine-rich domains separated by a cysteine-rich region (51). The rest of the β-subunit is composed of the transmembrane region and the C-terminal intracellular domain (52). The C-terminal intracellular domain consists of a tyrosine kinase catalytic domain that is flanked by two regulatory regions, which contribute to IGF-1R activation. The first regulatory region is a juxtamembrane site, which is involved in the docking of insulin receptor substrates (IRS) and Src homology collagen (Shc) and the internalization of the IGF-1R (53). The second regulatory region is a 108-residue carboxyterminal tail that contains phosphotyrosine binding sites (54). Figure 2 depicts IGF-1R's mRNA structure, its processing and its protein structure.

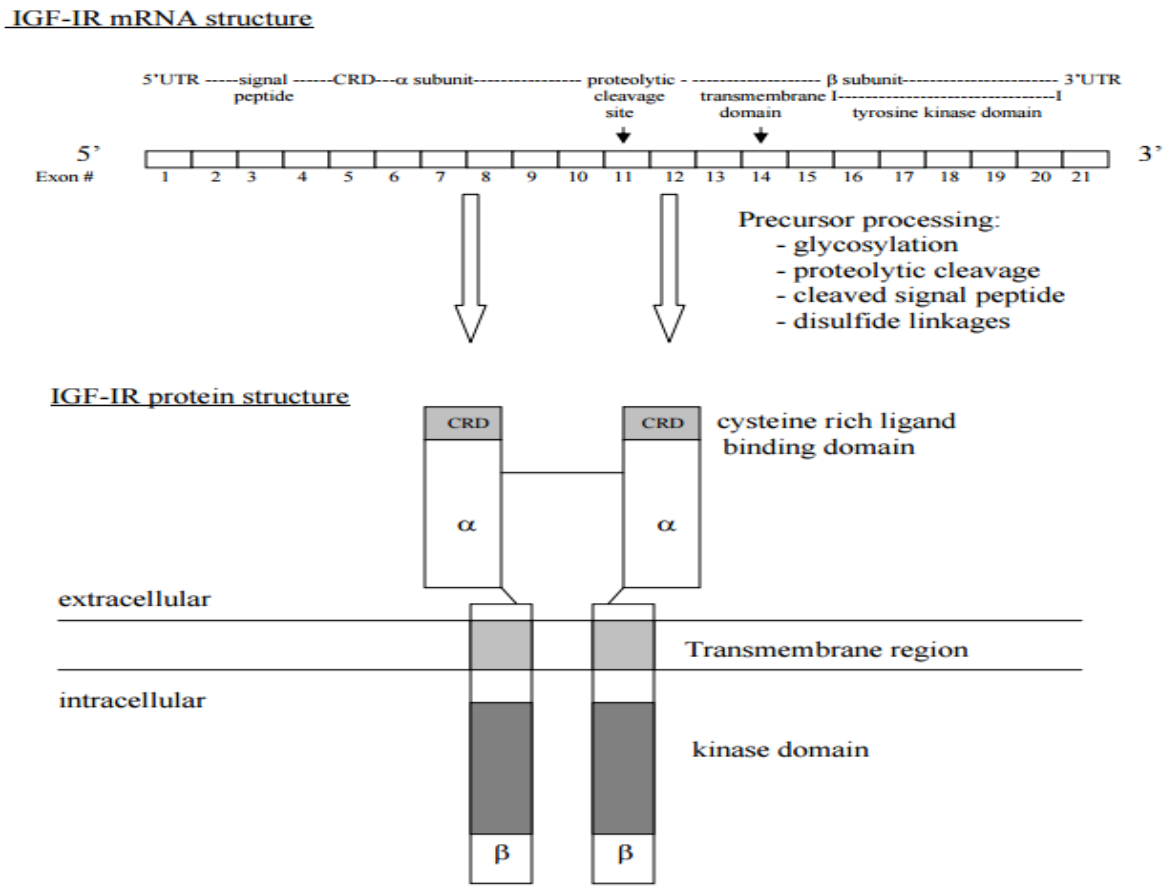


**Figure 2: IGF-1R mRNA structure, processing and protein structure. IGF-1R's functional domains are indicated above corresponding to numbered exons. Upon translation, the prepeptide is processed by means of glycosylation, proteolytic cleavage and addition of disulphide linkages. The α and β subunits are assembled and expressed on the cell membrane. The α subunit is exclusively extracellular and the β subunit is primarily intracellular, but it also contains a transmembrane region and a small extracellular region (47, 50-52).**

Once IGF-1 activates IGF-1R, its physiological effects are exerted through multiple signaling cascades, such as the mitogen activated protein kinase (MAPK) and the phosphatidyl-inositol-3-kinase/protein kinase B (PI3-K/PKB) cascades (47, 55, 56). The activation of these pathways is implicated in many cellular processes including VSMC growth, proliferation, migration and survival (57, 58). In terms of the specific events leading to IGF-1R activation, after IGF-1 binds to IGF-1R's extracellular α-subunit, the receptor undergoes a

conformational change, which activates the tyrosine kinase activity of the β-subunit. This then leads to the transautophosphorylation of several tyrosine residues in the regulatory domain of the β-subunit of IGF-1R (Tyr 1131, 1135 and 1136), resulting in increased receptor tyrosine kinase activity (59). IGF-1R's activated protein tyrosine kinase activity then phosphorylates several cytosolic substrates, which include IRS 1-4 and the adaptor protein Shc. These phosphorylated cytosolic substrates then serve as docking sites that bind with Src homology 2 (SH2) domain-containing proteins, such as growth factor receptor-binding protein 2 (Grb-2), the guanine nucleotide exchange factor son of sevenless (SOS) and the p85 regulatory subunit of PI3-K (60).

### 2.2. IGF-2 receptor

Unlike IGF-1R, IGF-2R, which is also called mannose-6-phosphate receptor, is a cell-surface receptor with no protein tyrosine kinase activity. IGF-2R is made up of 4 structural domains: an amino-terminal signal sequence domain, an extracytoplasmic domain, a transmembrane region and a carboxyterminal tail (61). IGF-2R functions in clearing IGF-2 from the cell surface by internalization and lysosomal degradation, thereby decreasing IGF-2's mitogenic effects (62). Furthermore, it has been shown that IGF-2R can exist in a cleaved form in circulation, therefore enabling it to act as an IGFBP for IGF-2 protein (62).

### 2.3. IGF-1 signaling

As previously mentioned, activation of IGF-1R by IGF-1 can activate multiple signaling pathways including the MAPK and the PI3-K/PKB cascades. The activation of these signaling

pathways induces different biological actions of IGF-1, including cell growth, migration, and survival. In addition to activating growth promoting signaling pathways, IGF-1 has also been shown to mediate its downstream effects through reactive oxygen species (ROS), namely superoxide and hydrogen peroxide, generation (15, 63, 64).

### 2.3A IGF-1 signaling and MAPK cascade

One of the signaling cascades activated by IGF-1 is the MAPK pathway. MAPKs, a group of serine-threonine kinases, are classically associated with cell growth, proliferation, differentiation and apoptosis, which are all key processes involved in VSMC remodelling (61). Five members of the MAPK family have been described in mammalian cells: extracellular signal regulated kinases 1 and 2 (ERK1/2), Jun N-terminal kinase 1, 2 and 3 (JNK1/2/3), p38 MAPK, ERK5, and ERK7 (65). These MAPKs all follow a similar system of sequential activation by several upstream signaling components, in which a stimulus activates a MAPKKK, which will then phosphorylate and activate a MAPKK, which will at last phosphorylate Thr and Tyr residues in the activation loop of the final effector, MAPK, leading to its activation and hence cellular response. For example, ERK1/2 activation by IGF-1 is initiated through the recruitment of the Grb-2/SOS complex to phosphorylated IRS or Shc. Subsequently, guanosine diphosphate (GDP) is exchanged for guanosine triphosphate (GTP), leading to the activation of Ras, a GTP-binding protein. This process initiates sequential phosphorylation by recruiting the serine-threonine kinase Raf to the membrane. This then leads to the activation of MAP kinase kinase 1 and 2 (MEK1 and MEK2) and ERK1/2 by phosphorylating threonine and tyrosine residues within a conserved Thr-Glu-Tyr motif in their activation loop (66, 67). Activation of ERK1/2 can lead to the phosphorylation of serine and threonine residues on downstream cytosolic targets of

ERK1/2. ERK1/2, along with other MAPK family members, can also be translocated from the cytosol to the nucleus where they can phosphorylate and activate several transcription factors, such as SRF, ATF2 and Jun, which are implicated in the activation of genes involved in cell growth and differentiation (68).

**2.3B IGF-1 signaling and PKB pathway**

Another well-known pathway that is triggered by IGF-1R activation in the vascular system is the PI3-K/PKB cascade. Similarly to the MAPK cascade, the PI3-K/PKB pathway plays a pivotal role in cell migration, differentiation, proliferation and survival (15).

PI3-Ks are grouped into three classes based on their substrate specificity, molecular structure and mode of regulation. Class I PI3-Ks generate phosphatidylinositol 3 phosphate (PI(3)P), phosphatidylinositol 3,4-bisphosphate (PI(3,4)P2) and phosphatidylinositol 3,4,5-triphosphate (PI(3,4,5)P3) and are activated by both R-PTK as well as G-protein coupled receptors (GPCR). Class II PI3-Ks generate PI(3)P and PI(3,4)P2, and possess a lipid binding domain, whereas, class III PI3-Ks only generate PI(3,4,5)P3 (69). Class I PI3-Ks are further subdivided into class IA and IB PI3-Ks and are heterodimeric proteins composed of a catalytic and regulatory subunit. Class IA proteins consist of a 110 kDa (p110) catalytic subunit and an associated 85 kDa (p85) regulatory subunit. Class IA has three isoforms of the catalytic subunit, p110α, p110β and p110δ, and several isoforms of the regulatory subunit, p85α, p55α, p50α, p85β and p55γ. In contrast to class IA, class IB has only one catalytic member, p110γ, and one form of the regulatory subunit, p101. The class IA PI3-Ks are activated by R-PTK, while class IB is activated by GPCR (70).

The p85 regulatory subunit of PI3-K, which is an SH2 domain-containing protein, interacts with phosphorylated IRS and stimulates the activation of p110 (69, 70). Activated PI3-K catalyzes the conversion of phosphatidylinositol 4,5-bisphosphate (PI(4,5)P2) to phosphatidylinositol 3,4,5-triphosphate (PI(3,4,5)P3), a key signaling molecule and important secondary messenger. PI(3,4,5)P3 recruits cytosolic proteins with pleckstrin-homology (PH) domains such as the serine/threonine kinase PKB and phosphoinositide-dependent kinase 1 and 2 (PDK1/2) (71).

Several downstream targets of PI3-K have been documented, the most studied being PKB, also known as Akt (a product of akt proto-oncogene). PKB is a 57 kDa serine-threonine kinase with three identified isoforms in the mammalian system, PKBα/Akt1, PKBβ/Akt2 and PKBγ/Akt3 (72). All isoforms possess an N-terminal pleckstrin homology (PH) domain that binds to phospholipids generated by PI3-K, a central catalytic kinase domain with specificity for serine or threonine residues on substrate proteins, and a C-terminal regulatory domain containing a hydrophobic motif (73). Full activation of PKB is a sequential two-step process where the lipid products generated from PI3-K are recognized by the PH domain of PKB, translocating it to the plasma membrane, where it then gets phosphorylated on Thr308 by PDK-1 and on Ser473 by mTORC2 for its complete activation (74). PKB has numerous downstream targets, such as mammalian target of rapamycin (mTOR) (72), glycogen synthase kinase-3 (GSK-3) (75), and caspase-9 (76). Figure 3 illustrates IGF-1-mediated activation of the MAPK and PKB cascades.

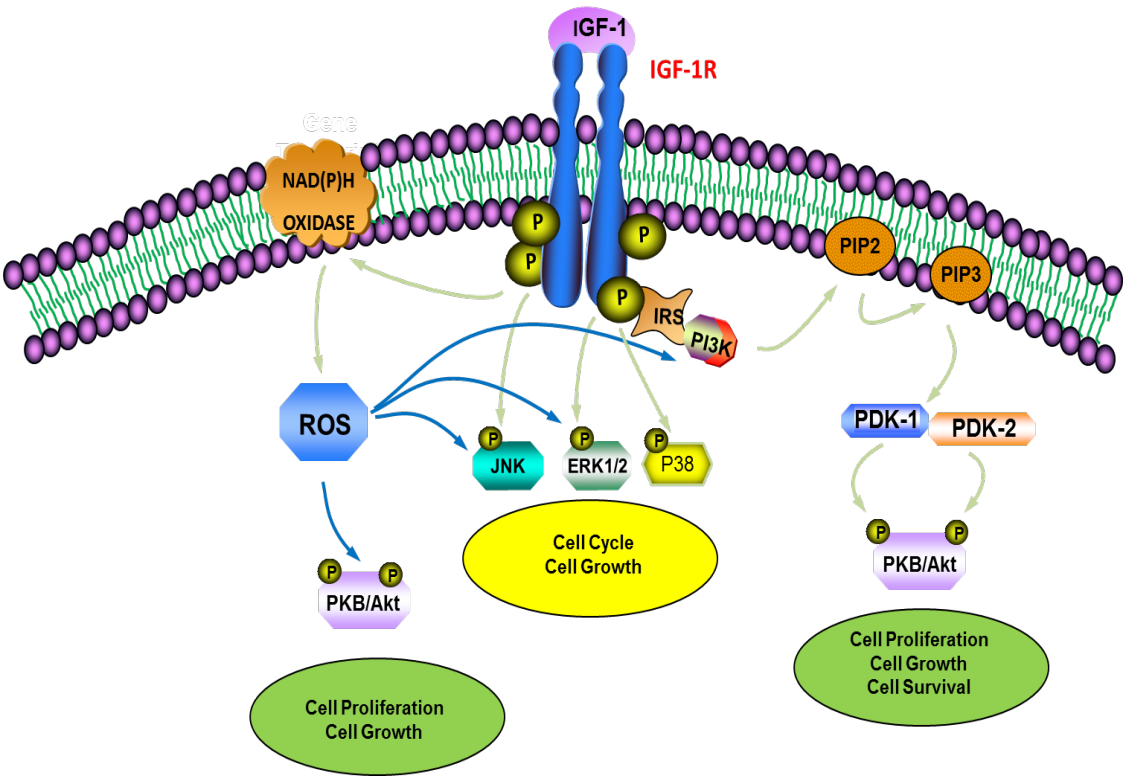


**Figure 3: Schematic representation of IGF-1 signaling pathways. IGF-1 functions as a high-affinity ligand for IGF-1R in the cellular membrane, leading to autophosphorylation of IGF-1R and recruitment of the IRS adaptor proteins. The interaction of IGF-1R with the IRS proteins induces the activation of PI3K. PI3K converts PIP2 into the lipid second messenger PIP3. PIP3 then activates PDK1 and PDK2, which go on to phosphorylate PKB at threonine 308 (Thr308) and serine 473 (Ser473), respectively. Activated PKB regulates downstream signaling molecules leading to cell proliferation and growth. In parallel, the activation of the MAPK pathway leads to increased cell survival, growth and cell cycle progression. Additionally, IGF-1R activation can lead to increased ROS generation via NAD(P)H oxidase activation, triggering the PKB and MAPK pathways (15, 61, 70, 71).**

### 2.3C IGF-1 signaling and ROS generation

As indicated in Figure 3, in addition to activating growth promoting signaling pathways, IGF-1 has also been shown to mediate its downstream effects through ROS generation (15). ROS are very small, rapidly diffusible, highly reactive molecules that take part in physiological

reactions as well as signal transduction, but excessive synthesis of these molecules can overcome their antioxidant mechanisms and generate deleterious effects, often resulting in the development of CVDs (77). This is in part due to their physicochemical properties which allow them to disrupt biological macromolecules such as lipids, DNA, carbohydrates and proteins. Hydrogen radicals ($OH^{\cdot}$), superoxide anions ($O2^{\cdot}$-), hydrogen peroxide ($H_2O_2$), reactive nitrogen radicals and its derivative peroxynitrite (ONOO-) are amongst the most important ROS. They are generated both enzymatically and non-enzymatically by nearly every cell type including VSMCs and endothelial cells (77). Studies have illustrated that the major source of ROS in VSMCs is NADPH oxidase (Nox), a flavoprotein (63, 64). It has been demonstrated that, in rat aortic VSMCs, the main components of the NADPH complex are the membrane-bound subunits of Nox and p22phox as well as Rac1, a small Rho-GTPase (63). A range of mediators detected in vascular diseases, including IGF-1, have been shown to modify Nox activity, leading to an increase in ROS production (63). Nox4 and Nox1 are the predominantly expressed isoforms of Nox in rat aortic VSMCs, however studies indicate that IGF-1 regulates the expression of Nox4 at a post-transcriptional level and, conversely, has little effect on the expression of Nox1 in VSMCs (63, 64). Inhibition of Nox4 expression by siRNA technology inhibits IGF-1-induced ROS generation in VSMCs. IGF-1-induced ROS generation has been shown to play an important role in VSMC proliferation and migration via MMPs, notably MMP-2 and -9 (63).

## 3. IGF-1 actions in the vasculature

IGF-1 exerts various actions in the vasculature. Numerous studies support the notion that IGF-1 is a potent mitogen that plays a permissive role in vascular dysfunction due to its pro-atherogenic properties, which contribute to VSMC proliferation and migration as well as neointimal formation (78, 79). Furthermore, IGF-1 has also been shown to promote chemotactic macrophage migration (80) and cell adherent molecule expression leading to the development of CVDs, such as atherosclerosis (58). On the other hand, IGF-1 has also been suggested to have a protective role in the vasculature.

### 3.1 Protective actions of IGF-1 in the vasculature

Evidence suggests that IGF-1 serves in endothelial repair and regulation of vascular tone under pathological conditions. It has been shown that IGF-1 induces vasorelaxation through the upregulation of nitric oxide (NO) production by stimulating inducible NO synthase (iNOS) in VSMC as well as through the activation of endothelial NO synthase (eNOS) in endothelial cells (ECs) (81). In fact, a study involving IGF-1 supplementation to diabetic patients, characterized by impaired vascular tone, resulted in improved vasodilatory responses mainly by increasing eNOS activity (58). Furthermore, studies using a pharmacological blocker of NO synthase (N-nitro-L-arginine methyl ester), suggest that IGF-1 acts on vascular tone by stimulating endothelial NO generation (82, 83). Moreover, studies using ECs have demonstrated that IGF-1 mediates NO production in an eNOS-dependent manner; this process also depends on the activation of the PI3-K/PKB pathway involving Ser1177 phosphorylation (58, 84).

In addition to its effects on vascular tone, high levels of IGF-1 in the intimal layer could potentially act as a potent survival factor preventing apoptosis of VSMCs, which assists in maintaining the structural integrity of the fibrous cap. This VSMC accumulation may in turn act as a vascular repair mechanism. Therefore, this protective role of IGF-1 may be beneficial for stabilization of the atherosclerotic plaque in the early stage of atherosclerosis (85).

Furthermore, IGF-1 has also been shown to exert anti-inflammatory and antioxidant properties by modulating cytokine responses and by upregulating eNOS activity. Using an ApoE-null mouse model, it was shown that IGF-1 infusion to the aorta reduced macrophage infiltration within atherosclerotic lesions, decreased aortic expression of pro-inflammatory cytokines and suppressed vascular superoxide levels via an increase in eNOS activity and NO bioavailability (86, 87). IGF-1R deficiency in the macrophages of ApoE-null mice was also shown to be associated with accelerated atherosclerosis (88). Furthermore, an in vitro study using cultured human aortic endothelial cells also found that IGF-1 has potent endothelial antioxidant effects. This effect is largely mediated through the upregulation of glutathione peroxidase-1 (GPX-1) expression and activity (89).

### 3.2. IGF-1 and vascular pathophysiology

In response to vascular injury, VSMCs downregulate the genes necessary for smooth muscle contractile function, such as α-smooth muscle actin and smooth muscle myosin heavy chain, they increase their proliferation rate and migration capacity, and they actively secrete matrix proteins (90). An upregulation of genes associated with the proliferative state, such as

KLF4 and PDGFR (91, 92), also occurs. Furthermore, a change in the expression of calcium (Ca2+) signaling proteins, such as voltage-gated Ca2+ channels, ryanodine receptors, STIM1/Orai1 channels and SERCA pump isoforms (93), is seen in this process, affecting Ca2+ excitation-transcription coupling in VSMCs. This state is called the "activated" or "synthetic" state. Although the synthetic state is required for vascular development and during vascular repair, it plays a pivotal role in the development of multiple smooth muscle diseases, such as atherosclerosis and restenosis following angioplasty (94). IGF-1 has been associated with mediating the VSMC phenotype switch from the quiescent/contractile state to the activated/synthetic state. Therefore, in pathological conditions, for example in the early stage of atherosclerotic plaque formation, a decrease in circulating IGF-1 levels could be beneficial (95).

**3.2A IGF-1 and atherosclerosis**

Atherosclerosis is an inflammatory process, which occurs when VSMCs proliferate and migrate to the intima layer resulting in neointima formation and the subsequent development of atherosclerotic lesions (96). IGF-1's involvement in the pathogenesis of atherosclerosis has been supported by several studies, which collectively indicate a marked increase in IGF-1 and IGF-1R expression in human atherosclerotic plaques and also demonstrate that IGF-1 is able to stimulate VSMC migration and proliferation (97, 98). Studies using various inhibitors have shown that a decrease in IGF-1 or IGF-1R signaling in VSMCs is associated with reduced neointimal area size, suggesting that IGF-1 possesses a role in promoting vascular hyperplasia. Similarly, targeted overexpression of IGF-1 in VSMCs from carotid arteries resulted in an increase in neointimal formation (99). Despite the fact that, as mentioned above, studies in animal models and human subjects (86-89) have demonstrated IGF-1's anti-inflammatory effects, there are

studies that suggest otherwise. A possible pro-inflammatory role of IGF-1 in early pro-atherosclerotic lesions was suggested by Che et al by demonstrating that IGF-1 increased basal TNF-α mRNA expression and production in cultured bovine endothelial cells (100). Moreover, in atherosclerotic macrophages, it has been illustrated that IGF-1 favors excess LDL uptake and cholesterol esterification as well as the release of inflammatory cytokines and upregulated chemotactic macrophage migration (58, 80).

### 3.2B IGF-1 in hypertension

Several studies have found a link between IGF-1 and hypertension. For example, increased IGF-1 mRNA expression has been found in rat models of aorta hypertrophy, urinary bladder hypertrophy and portal vein hypertrophy (101). In spontaneously hypertensive rats (SHR), increased IGF-1 caused impaired vasorelaxant properties in pre-contracted norepinephrine thoracic aortic rings as compared to normotensive (Wistar Kyoto Rats) rings (102). Furthermore, a marked increase in arterial pressure was observed in homozygous mice containing a site-specific mutation in IGF-1's exon 3 (103), further implicating IGF-1 in the regulation of blood pressure. IGF-1's involvement in blood pressure regulation was also observed by another group who studied liver-specific IGF-1 knockout mice and saw an increase in peripheral resistance and systolic blood pressure in these mice (104). Finally, studies performed in patients with essential hypertension reported elevated levels of IGF-1, further suggesting a role of IGF-1 in hypertension development (98, 105). Despite these discoveries, the precise role of IGF-1 in hypertension still remains unclear.

## 4. IGF-1 and histone modifications

HDACs and histone acetyltransferases (HATs) regulate gene expression through the removal or addition, respectively, of acetyl groups on lysine residues of core nucleosomal histones. Increased histone acetylation attenuates the electrostatic interaction between histones and the negatively charged DNA backbone, thereby promoting the unfolding of the histone–DNA complex. This then enables transcription factors to access their sites of action, modulating the transcription of their target genes (106). As described above, IGF-1 participates in the progression of vascular remodeling by activating growth promoting and proliferative signaling pathways, such as the MAPK and PKB pathways. The activation of these pathways has been shown to alter the acetylation of status of histone and non-histone proteins (107) therefore it is possible that IGF-1 may contribute to the development of CVDs by modulating the activity of HDACs and HATs.

**4.1 General overview of histone modifications**

In eukaryotic cells, to accommodate the large mass of genetic material within the nucleus, DNA is packaged into chromatin. Chromatin is composed of a combination of DNA, histone proteins and some RNA. Histones can be covalently modified by a variety of processes including acetylation, methylation, SUMOylation, ubiquitination and citrullination (108), thereby providing a regulatory mechanism for controlling gene expression. These modifications alter the structure of the DNA-histone complex, which in turn affects DNA transcription. Each of these histone modifications may be involved in epigenetics; however acetylation has been shown to be particularly important in CVDs (109). As mentioned above, acetylation of lysine residues neutralizes the charge on histones, which increases chromatin accessibility (109). This is

achieved by HATs, which attach acetyl groups to conserved lysine residues on histone tails. Conversely, HDACs remove these groups and therefore reduce DNA transcription. Several studies suggest the involvement of HDACs in vasoactive peptide/growth factor-mediated aberrant VSMC proliferation and hypertrophy, linking HDACs to vascular pathophysiology (110,111,112).

**4.2HDAC system and classes**

As mentioned above, HDACs are a group of enzymes that lead to deacetylation of lysine residues on histones. They have also been shown to deacetylate non-histone proteins as well (113). At least 18 different mammalian HDAC genes have been identified, which can be categorized into four classes based on structure, function and the associated yeast orthologue. The four classes are class I HDACs (HDAC1, 2, 3 and 8), class II (HDAC4, 5, 6, 7, 9, 10), class III HDACs (sirtuins), and class IV HDAC (HDAC11). Classes I, II and IV HDACs have been termed classical HDACs as they require zinc as a cofactor, while class III HDACs require NAD+ as a cofactor (114). Class II HDACs are further subdivided into class IIa (HDAC 4, 5, 7 and 9) and class IIb (HDAC 6 and 10) based on their primary structure. In contrast to class IIb HDACs, class IIa HDACs contain a large N-terminal regulatory domain involved in protein-protein interactions, in addition to a C-terminal catalytic domain (115). Class IIa HDACs seem to have critical roles in many disease processes, including CVDs. For example, HDAC4 and HDAC5 have been shown to be critical in promoting VSMC proliferation and migration in response to growth factors and vasoactive peptides (110, 111, 116) and HDAC7 has been linked to PDGF-BB-induced endothelial cell migration (117).

### 4.3HDAC localization

HDAC localization, which can either be nuclear or cytoplasmic, greatly affects HDAC activity. HDACs that are able to shuttle in and out of the nucleus have been shown to regulate various cytoplasmic processes and function as signal transducers (118). Localization varies depending on the class. Class I HDACs are primarily located in the nucleus with the exception of HDAC3, which can also translocate into the cytoplasm. In contrast, class II HDACs are able to shuttle in and out of the nucleus. Some Class III and IV HDACs can also shuttle between the nucleus and cytoplasm, while others are confined to either the nucleus or the cytoplasm (114). Table 1 summarizes the HDAC classes, the members of each class and their localization.

| HDAC class | Members | Localization |
|---|---|---|
| Class I | HDAC 1 | Nucleus |
| | HDAC 2 | Nucleus |
| | HDAC 3 | Nucleus/Cytoplasm |
| | HDAC 8 | Nucleus |
| Class II | HDAC 4 | Nucleus/Cytoplasm |
| | HDAC 5 | Nucleus/Cytoplasm |
| | HDAC 6 | Nucleus/Cytoplasm |
| | HDAC 7 | Nucleus/Cytoplasm |
| | HDAC 9 | Nucleus/Cytoplasm |
| | HDAC 10 | Nucleus/Cytoplasm |
| Class III | SIRT1 | Nuclear |
| | SIRT2 | Cytoplasm |
| | SIRT3 | Nucleus/Cytoplasm |
| | SIRT4 | Cytoplasm |
| | SIRT5 | Cytoplasm |
| | SIRT6 | Nuclear |
| | SIRT7 | Nuclear |
| Class IV | HDAC 11 | Nucleus/Cytoplasm |

**Table 1: HDAC classes and localization. This figure illustrates the four HDAC classes, the members of each class and their localization (113, 114).**

### 4.4 HDAC structure

Despite the fact that it has been over 50 years since Allfrey and coworkers first proposed the idea that the acetylation status of the histone proteins that make up chromatin is correlated with the transcriptional status of a given gene (119), information regarding HDAC structure has only become available more recently. Due to their comparable enzymatic activities, it would be expected that the proteins within the HDAC superfamily share some structural features. A study of the available structural information reveals that this is indeed true. In humans, HDACs are divided into separate classes based on sequence similarities. The Class I proteins (HDAC1, HDAC2, HDAC3, HDAC8) have a sequence similarity that extends over 300 residues with the yeast Rpd3 protein. Within this 300 residue sequence, there is an especially marked homology within an internal ~70 residue stretch (120). To be specific, 80% similarity was found between yeast Rpd3 and human HDAC1 within the 300 residue region of conservation and 99% homology in the 70 residue stretch (120). The Class II HDAC proteins (HDAC4, HDAC5, HDAC6, HDAC7, HDAC9 and HDAC10) have a comparable sequence with the yeast Hda1 protein. Additionally, mammalian Class I and II HDACs were also found to be related to *Saccharomyces cerevisiae* Hos1, Hos2 and Hos3 proteins, which have 35-49% identity to Rpd3 and 21-28% identity to Hda1 (121). The Class III proteins (SIRT1, SIRT2, SIRT3, SIRT4, SIRT5, SIRT6, and SIRT7) and the yeast Sir2 protein have a comparable sequence (113). The Class IV protein (HDAC11) has a comparable sequence to both Class I and II proteins (113). Figure 4 shows domain organization of human HDACs (113).

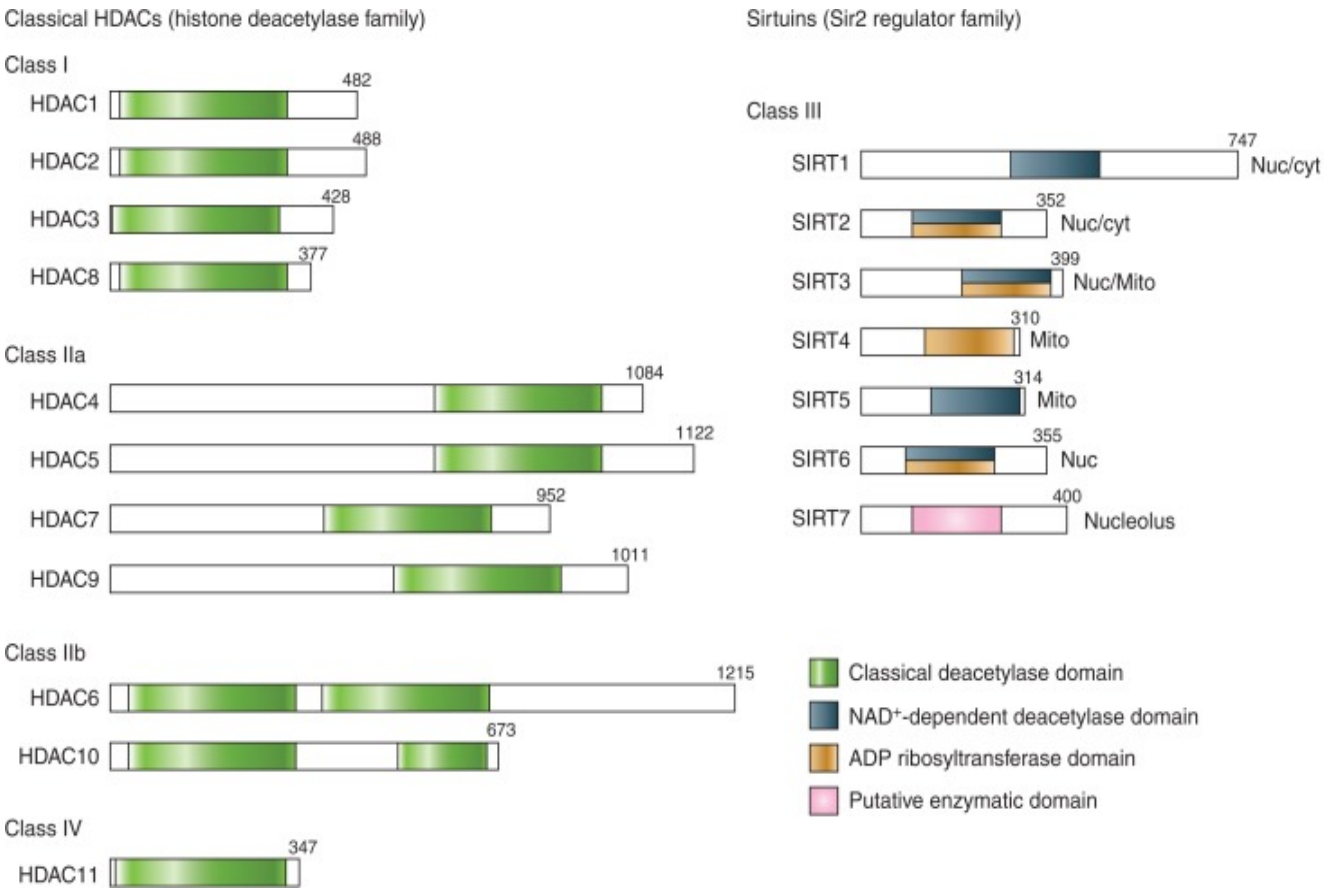


**Figure 4: This figure illustrates domain organization of human HDACs. The total number of amino acid residues in each HDAC is shown. Also, only the longest isoform is shown for simplicity, despite the fact that HDACs have several isoforms. Enzymatic domains (or putative enzymatic domains) are shown in colors (113).**

Class I and II HDACs have some structural similarities to Class III HDACs in that they all contain a central parallel β sheet network that is flanked on opposite sides by helix-rich segments (120). Moreover, sequence conservation studies suggest that, in all three HDAC classes, substrate binding and catalysis is mediated by the domain proximal to the C-terminal tip of the β sheet. This domain is rich in loop segments that appear to be critical for function as they form protein cavities that are implicated in acetyl-lysine binding (122). Despite these marked

similarities, there are also striking structural differences between the Class I/II and Class III HDACs. Most notably is the small globular region in Class III HDACs, which is absent in Class I/II HDACs. This region is important for class III HDAC deacetylase activity and, due to its sequence and structural divergence, it plays a modulatory role in substrate specificity within the class III HDAC family (120). The absence of a corresponding domain in Class I/II HDACs suggests that other proteins may substitute for this domain in vivo. This is consistent with the observation that Class I/II HDACs associate with other proteins in vivo for catalytic activity (120).

## 4.5. HDAC expression and regulation

### 4.5.1 Class I HDACs

In terms of HDAC expression and regulation, Class I HDACs are ubiquitously expressed nuclear enzymes; however, it has been shown that HDAC8 is generally poorly expressed (118). Not a great deal is known about HDAC3 and HDAC8 regulation, but the other Class I HDACs (HDAC1 and HDAC2) form homo- and heterodimers between each other (123,124), which is a requirement for HDAC activity. Dissociation of the dimer will impede HDAC activity, which is something viruses take advantage of to hinder HDAC activity. For example, it has been shown that, by binding to the N-terminal region of HDAC1 and likely dissociating the dimer, the adenoviral protein GAM1 impedes HDAC1 activity (125). The level of HDAC1 and HDAC2 heterodimers is likely cell type-specific, because it was demonstrated that 80% to 90% of HDAC1 and HDAC2 proteins formed dimers in the nucleus of human breast cancer MCF-7 cells (126), whereas, in mouse embryonic fibroblasts, 40% to 60% of HDAC1 and HDAC2 proteins were shown to be free from each other (127). Additionally, Class I HDACs depend on the

presence of a catalytic Zn2+ ion for their activity and have been shown to be sensitive to inhibition by a family of small molecule compounds that have homology to trichostatin (TSA) such as suberoylanilide hydroxamic acid (SAHA) and trapoxin (TPX) (128).

#### 4.5.2 Class II HDACs

Like Class I HDACs, Class II HDACs also depend on the presence of a catalytic Zn2+ ion for their activity. Class II HDACs, as previously mentioned, shuttle between the nucleus and cytoplasm and have tissue-specific expression and functions (128).

#### 4.5.2a Class IIa HDACs

Class IIa HDACs (HDAC4, HDAC5, HDAC7 and HDAC9) are important signal transducers. In the regulatory N-terminal domain of these HDACs, there are several conserved serine residues that are subject to reversible phosphorylation. An array of kinases and phosphatases acting downstream of diverse biological pathways have been demonstrated to act on these HDACs, regulating their nucleocytoplasmic trafficking. On HDAC4, this phosphorylation occurs on serine 246 (S246), S467 and S632 by several isoforms of the calcium/calmodulin-dependent kinase (CaMK) family (129, 130), by Aurora B on S265 (131), by Mirk/dtrk1B on S266 (132), and by glycogen synthase kinase 3β (GSK3β) on S298 and S302 (133). The critical residues on HDAC5 are S259 and S498 and have been shown to be phosphorylated by CaMK-1, -II and –IV, protein kinase D (PKD) and AMPK (134,135,136). Protein kinase C (PKC), an upstream regulator of PKD, has also been shown to phosphorylate S259 directly (137). For HDAC7, the critical residues are S181, S155, S358 and S486 (138). The latter three sites can be modified by CaMK1, whereas PKD can phosphorylate all four residues.

Less is known about HDAC9, but it has been suggested that HDAC9 residues S239, S240 and S253 are phosphorylated by Aurora B, Mirk/dtrk1B and PRK1, respectively (138). Class IIa HDAC phosphorylation leads to the binding of 14-3-3 proteins, the nuclear export of these HDACs and the de-repression of their target genes (139).

#### 4.5.2b Class IIb HDACs

Class IIb HDACs (HDAC6 and HDAC10) have duplicated catalytic domains, however the duplication is only partial in the case of HDAC10 (140). HDAC6 and HDAC10 shuttle between nucleus and cytoplasm, but their location is primarily cytoplasmic (141). Not much is known regarding class IIb HDAC regulation and expression.

### 4.5.3 Class III HDACs

Class III HDACs, also known as sirtuins (SIRT1, SIRT2, SIRT3, SIRT4, SIRT5, SIRT6, SIRT7), are NAD+ dependent and the availability of $NAD^+$ in cells is a limiting step in the activation of sirtuin catalytic activity (142). The basal intracellular NAD+ levels are maintained relatively constant (143) through NAD+ biosynthetic and salvaging pathways (144). By consuming $NAD^+$ in order to exert their effects, sirtuins regulate the fluctuation of the $NAD^+$/NADH ratio. Additionally, sirtuin gene expression has been shown to be under the control of numerous transcription factors involved in cell cycle regulation and apoptosis. Among them the transcription factor E2F1, which induces cell cycle progression from G1 to S phase, directly binds to the Sirt1 promoter upregulating its gene expression in cells treated with the

topoisomerase II inhibitor etoposide (145). Furthermore, the tumour suppressor p53, which is one of the most extensively mutated proteins in cancers, has also been shown to affect Sirt1 gene expression. Two functional p53-binding sites have been identified in the regulatory region of the Sirt1 promoter and studies have indicated that in nutrient-deprived mammalian cells p53 stimulates Sirt1 gene expression (146). On the contrary, in normal nutrient conditions, p53 mediates repression of Sirt1 gene expression (147). Sirt1 levels are regulated by E2F1 and p53 at the transcriptional level as well as the translational level.

Sirtuin activity is also regulated by posttranslational modifications. *In vitro* evidence has demonstrated that cyclin B/Cdk1-mediated dephosphorylation at specific sites decreases Sirt1 and Sirt2 deacetylase activity (148). Additionally, Sirt1 has also been shown to be phosphorylated by the c-Jun N-terminal kinase 2 (JNK2) (149) and casein kinase 2 (CK2) (150). JNK2-mediated phosphorylation of Sirt1 is associated with the regulation of its protein stability (149). Furthermore, several conserved phosphorylation sites have been found within Sirt1 that are possible targets for a variety of kinases such as ATM, casein kinase 1 (CK1), DNA-dependent protein kinase (DNA-PK), extracellular-signal-regulated kinase (ERK1), GSK-3, I$\kappa$B kinase (IKK), and MAPK (148). It is not known, however, if these kinases phosphorylate only Sirt1 or other members of the sirtuin class as well. In addition to phosphorylation, other posttranslational modifications that may regulate sirtuin deacetylase activity include acetylation and sumoylation.

#### 4.5.4 Class IV HDACs

The Class IV HDAC (HDAC11) has sequence similarity to Class I and II HDACs. Aside from this, little is known of its function and regulation (113).

## 4.6. HDAC activation and signaling in vascular pathophysiology

### 4.6A Vasoactive peptide-induced HDAC activation and signaling

Angiotensin II (AngII), a potent vasoconstrictor, has been shown to activate several signaling pathways linked to cellular hypertrophy, growth, migration and proliferation in various cell types including VSMCs. One of its targets is CaMKII, which transduces downstream signaling responses of AngII upon activation (151). A potential transcriptional target for CaMKII is myocyte enhancing factor 2 (MEF2). MEF2, a DNA binding transcription factor which likely promotes the synthetic phenotype of VSMCs, can either act as an activator or a repressor of transcription depending on its interaction with co-activators or co-repressors, respectively (152). HDAC4 and HDAC5 have been shown to directly interact with MEF2 in the nucleus to promote its repressive activity. Consequently, HDAC4 and HDAC5 phosphorylation and nuclear export increases MEF2 transcriptional activity and leads to VSMC hypertrophy (152). As recently reported, CaMKII is capable of mediating AngII-dependent increases in HDAC4 (S467) (153) and HDAC5 (S498) (154) phosphorylation and subsequent nuclear export. This derepresses MEF2, increasing MEF2 DNA binding activity and transcription. Additionally, it was demonstrated that HDAC5 phosphorylation is mediated by HDAC4 in VSMCs, suggesting a potential regulatory mechanism involving protein-protein interaction (154). Figure 5 illustrates CaMKII-dependent nuclear export of HDAC4 and HDAC5.

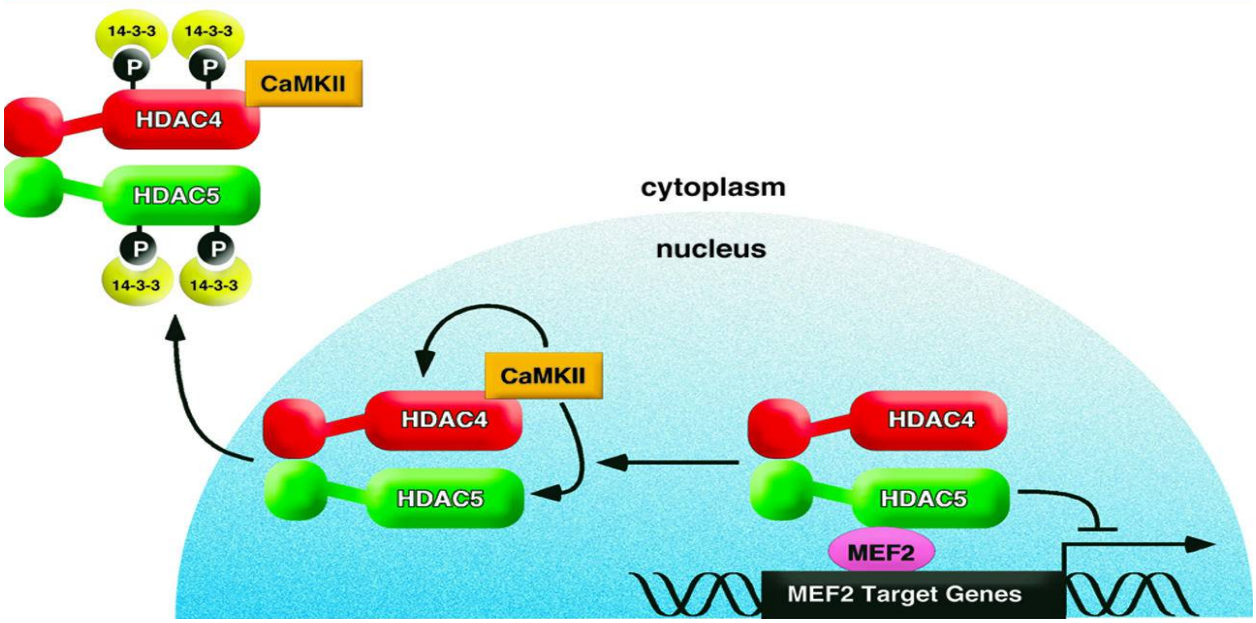


**Figure 5: This model depicts CaMKII-dependent nuclear export of HDAC4 and HDAC5. Following AngII stimulation, CaMKII specifically phosphorylates HDAC4 at S467 and HDAC5 at S498 (sites not shown in model), resulting in 14-3-3 protein-mediated nuclear export. Following HDAC4 and HDAC5 nuclear export, MEF2 is derepressed and MEF2-dependent gene transcription is activated (154).**

Based on evidence from different cell types, class IIa HDACs have been shown to have many transcription factor targets in addition to MEF2, including serum response factor (SRF), which has been linked to CaMKII and HDAC4 in cardiomyocytes (155). Davis et al. showed that HDAC4 interacts with SRF in cardiomyocytes and this interaction is enhanced by AngII stimulation (155). Thus, MEF2 is unlikely to be the only transcription factor targeted by CaMKII and class IIa HDACs in VSMCs.

As suggested above, there are many pathways and factors involved in propagating hypertrophic and proliferative signals in VSMCs. As evidenced by Pang et al., G-protein coupled

receptor (GPCR)-kinase 2 interacting protein-1 (GIT1) has been shown to be involved in mediating HDAC5 phosphorylation by AngII (156). AngII stimulation leads to GIT1 phosphorylation, causing PLCγ activation which is required for elevation of intracellular Ca2+ and activation of CaMKII. Once CaMKII is activated, it phosphorylates HDAC4 and HDAC5 leading to nuclear export (152).

In contrast to CaMKII-dependent HDAC4 and HDAC5 phosphorylation, other studies have shown that AngII is able to induce HDAC5 phosphorylation and nuclear export in VSMCs in a calcium independent manner (157). As evidenced by Xu et al., AngII has been shown to induce HDAC5 phosphorylation and export via the protein kinase C (PKC)-protein kinase D1 (PKD1) pathway. PKD1 has been shown to phosphorylate HDAC5 at Serine 259/498 (157). PKC has also been shown to phosphorylate Serine 259 directly in the tissue of failing hearts (158). Once phosphorylated, these residues serve as docking sites for 14-3-3 chaperone proteins. This results in an increase in MEF2 transcriptional activity, which derepresses VSMC growth genes and consequently leads to VSMC hypertrophy (116).

#### 4.6B Growth factor-induced HDAC phosphorylation and activation

In addition to vasoactive peptides, like AngII, growth factors are also involved in HDAC-related vascular pathophysiology. In VSMCs, HDAC4 and HDAC5 have been shown to be phosphorylated by CaMKII in response to Platelet-Derived Growth Factor-BB (PDGF-BB) (159).

PDGF-BB is a key mediator of VSMC phenotype switching from the contractile state to the synthetic state (160). As previously described, this switch is associated with suppressed expression of VSMC marker genes, such as smooth muscle α-actin and smooth muscle myosin heavy chain, as well as increased proliferation and migration rates of cultured VSMCs. It has been shown by Yoshida et al. that PDGF-BB represses the expression of VSMC marker genes through the recruitment of HDAC4 and HDAC5 to the promoters of these genes (161). HDAC-induced hypoacetylation inhibits the accessibility of transcription factors, notably SRF, to interact with the promoters of these genes, reducing transcription (162). Additionally, HDAC5 has been shown to directly interact and inhibit myocardin, SRF's coactivator, which also contributes to decreased transcription of marker genes (163). In terms of proliferation and migration, PDGF-BB- induced HDAC4 phosphorylation is involved in mediating proliferation and migration of VSMCs (164). HDAC4 knockdown was shown to inhibit PDGF-induced expression of cyclin D1, a cell cycle regulatory protein required for the progression of the G1 phase, which has an inhibitory effect on proliferative signals (165). Furthermore, Usui et al. also illustrated the involvement of HDAC4 in PDGF-BB-induced VSMC migration and cytoskeletal reorganization (164). Both migration and cytoskeletal reorganization were shown to be significantly inhibited by HDAC4 siRNA as well as MC1568, a Class IIa HDAC inhibitor (164).

In addition to VSMC phenotype switching, it is likely that PDGF-BB also increases ROS production by upregulating NOX activity in an HDAC4-dependent manner, as evidenced by

HDAC4 siRNA studies (166). Once HDAC4 is phosphorylated by PDGF-BB in a CaMKII-dependent manner, it is believed that HDAC4 might upregulate NOX activity via p47 phox or Rac-1 activation (167). Upregulation of NOX activity will cause increased ROS, which can stimulate VSMC proliferation and migration via the activation of p38MAPK/HSP27 signals (167).

In terms of the specific signaling cascades activated during PDGF-BB-mediated vascular pathophysiology, CaMKs and PKC/PKD are known to be activated by PDGF-BB leading to VSMC migration and proliferation (168, 169). HDAC4 has been shown to be a substrate for activated CaMKII (170). It has been suggested that PDGF-BB stimulation causes CaMKIIδ to sequester HDAC4 to the cytoplasm, thereby stimulating MEF2 activity in VSMCs (152).

#### 4.6C Prostanoids and B-adrenergic agonists-induced HDAC activation and signaling

In contrast to vasoactive peptides and growth factors, which contribute to vascular disease, prostanoids, such as prostacyclin, and β-adrenergic agonists decrease cardiovascular risk (171). Prostacyclin and β-adrenergic agonists activate protein kinase A (PKA), which has been shown to minimize the incidence of cardiovascular disease by opposing VSMC proliferation (171). Although the mechanisms underlying this process have not yet been fully elucidated, PKA has been shown to promote HDAC4-induced repression of MEF2-dependent gene expression (172).

### 4.7. Involvement of HDACs in cardiovascular pathophysiology

The majority of CVDs, such as hypertension and atherosclerosis, are the result of vascular remodeling mediated in part by VSMC proliferation and migration. HDACs, notably Class IIa HDACs, have been associated with this process (173).

### 4.7A Hypertension

HDAC4 is of particular importance in disease as it has been linked to the promotion of hypertension. Proliferation and migration of VSMCs leads to medial thickening and structural remodeling, also known as neointimal hyperplasia. Neointimal hyperplasia is one of the major processes underlying hypertension development. Through the use of MC1568, a Class IIa HDAC inhibitor, it was suggested that HDAC4 is at least partly responsible for neointimal hyperplasia and, consequently, has a role in hypertension development (174).

Vascular inflammation is also known to be important in the pathogenesis of hypertension. In particular, inflammation induced by ROS has been suggested to have a critical role in the development of hypertension via promoting proliferation and migration of VSMCs (175). Class IIa HDACs have been linked to this process and HDAC4 is of particular importance. It has been demonstrated that HDAC4 promotes ROS-dependent vascular inflammation and likely mediates the further development of hypertension in spontaneously hypertensive (SHR) rats (176). In these rats, HDAC4 has been shown to mediate ROS-dependent VSMC proliferation and migration through the activation of p38 MAPK/ HSP27 (176).

### 4.7B Atherosclerosis

VSMCs undergo a switch from the contractile to the synthetic phenotype in response to various stimuli, such as shear stress, lipids, ROS and cytokines, giving rise to a state favoring migration and proliferation (177). VSMCs recruited from the media to the intima, as well as those proliferating within the intima, contribute to progression of atherosclerosis. They secrete large quantities of extracellular-matrix components, such as collagen, resulting in atherogenic lipoprotein retention and aggregation (178). Additionally, migrating and proliferating smooth muscle cells beneath the endothelium form a fibrous cap, cutting the blood supply from the plaque (179). Thus, it is evident that smooth muscle cell proliferation and migration are key events in the development of atherosclerosis. HDACs are implicated in this process as studies have shown that they play a critical role in the VSMC phenotype switch needed for the progression of atherosclerosis. Class IIa HDACs have been shown to promote the phenotype switch of smooth muscle cells though Ca2+ signals (180). As previously mentioned, in addition to the phenotypic switch, VSMC migration and proliferation are also integral to the development of atherosclerotic vascular disease. Studies have indicated that HDAC4 and HDAC5, following phosphorylation by PKD1 and CaMKII, promote PDGF-BB-induced VSMC proliferation and migration via activating the p38 MAPK/HSP27 pathways, ultimately contributing to neointimal hyperplasia, vascular remodeling and the atherosclerotic plaque formation (181,182).

## 5. Goal of this study

IGF-1, a potent mitogenic and vasoactive factor, has been shown to play a role in the development of CVDs. This occurs through the hyperactivation of mitogenic and growth promoting signaling pathways and the subsequent alteration in gene expression induced by IGF-1. HDACs, by their ability to modify the acetylation status of the lysine residues in histone and non-histone proteins, regulate the transcription of genes involved in cell growth, proliferation and migration. Recent studies have demonstrated that a heightened activation of HDACs, notably HDAC5, is associated with vascular disorders such as atherosclerosis. However, a role of IGF-1 in HDAC activation by phosphorylation remains poorly characterized. More importantly, despite the fact that IGF-1 receptor activation leads to a potent stimulation of the ERK1/2 and PKB pathways, the involvement of these pathways in HDAC phosphorylation and nuclear export has not been explored in VSMCs. Therefore, in the present studies, we examined the effect of IGF-1 on the phosphorylation and nuclear export of HDAC5 in VSMCs and identified the signaling pathways involved in this process.

# CHAPTER 2

# ARTICLE

**PKB mediates Insulin-Like Growth Factor 1-induced phosphorylation and nuclear export of Histone Deacetylase 5 via NADPH Oxidase 4 activation in vascular smooth muscle cells**

**To be submitted to**

# PKB mediates Insulin-Like Growth Factor 1-induced phosphorylation and nuclear export of Histone Deacetylase 5 via NADPH Oxidase 4 activation in vascular smooth muscle cells

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# Abstract

Insulin-like growth factor 1 (IGF-1), a potent mitogenic and vasoactive factor, has been shown to play a role in the development of cardiovascular diseases. This occurs through the generation of reactive oxygen species (ROS) as well as through the hyperactivation of mitogenic and growth promoting signaling pathways and the subsequent alteration in gene expression. Histone deacetylases (HDACs), by their ability to modify the acetylation status of the lysine residues in histone and non-histone proteins, regulate gene transcription. Recent studies have demonstrated that a heightened activation of HDACs, notably HDAC5, is associated with vascular disorders such as atherosclerosis. However, a role of IGF-1 in HDAC phosphorylation and activation has not been investigated. Therefore, in the present studies, we examined the effect of IGF-1 on the phosphorylation of HDAC5 in vascular smooth muscle cells (VSMCs) and identified the signaling pathways involved in this process. Treatment of A10 VSMCs with IGF-1 enhanced the phosphorylation of HDAC5 at serine 498 in a time and dose-dependent fashion. Pretreatment of cells with AG1024, a selective pharmacological inhibitor of IGF-1R, significantly inhibited IGF-1-induced HDAC5 phosphorylation in A10 VSMCs whereas AG1478, a selective inhibitor of epidermal growth factor receptor (EGFR), did not have an inhibitory effect on the levels of phospho-HDAC5. Pharmacological blockade of the MAPK pathway with PD98059, UO126, SP600125 and SB203580 had no effect on HDAC5 phosphorylation, whereas inhibitors of the PI3K/ PKB pathways, wortmannin and SC-66 respectively, almost completely attenuated IGF-1-induced HDAC5 phosphorylation. These findings were confirmed by immunofluorescence localization of phospho-HDAC5 and by siRNA- induced silencing of PKB. In addition, pretreatment of A10 VSMCs with Diphenyleneiodonium (DPI) and apocynin, two NAD(P)H oxidase inhibitors, as well as the antioxidant N-Acetyl-Cysteine (NAC), resulted in an attenuation of IGF-1-induced phosphorylation of HDAC5 as well as PKB. Furthermore, siRNA-induced silencing of Nox4, the main NADPH oxidase expressed in VSMC, inhibited IGF-1 induced HDAC5 phosphorylation. Moreover, IGF-1-induced phosphorylation of HDAC5 resulted in its nuclear export, which was reversed by blockade of PKB by SC-66 or NAD(P)H oxidase inhibition by DPI. In summary, these data demonstrate that IGF-1 induces the phosphorylation and nuclear export of HDAC5 in a Nox4-derived ROS- and PKB-dependent fashion in VSMCs.

**Keywords:** IGF-1, HDAC5, PKB, PI3-K, MAPK, ROS, VSMC

## Introduction

Cardiovascular diseases (CVDs) are associated with the increasingly high morbidity and mortality rates worldwide (1). The main feature of CVD is an increased vascular resistance due to blood vessel remodeling. Blood vessels are lined by vascular smooth muscle cells (VSMCs), which are highly specialized cells that regulate vascular tone and blood pressure (2). Abnormal migration, growth and proliferation of VSMCs are believed to be key events that contribute to structural remodeling of blood vessels and have been attributed to the pathogenesis of vascular diseases such as intimal hyperplasia, hypertension, atherosclerosis and restenosis (3-7). A potential role of vasoactive peptides and growth factors in inducing proliferation, hypertrophy, migration and differentiation of VSMCs has been demonstrated (8-11). We have shown earlier that transactivation of insulin-like growth factor 1 (IGF-1) receptor is critical to induce the mitogenic and hypertrophic responses of two key vasoactive peptides, angiotensin II (AngII) and endothelin-1 (ET-1) (12). Recent studies have demonstrated that a heightened activation of histone deacetylases (HDACs), notably HDAC4 and HDAC5, is associated with increased proliferation, migration and hypertrophy of VSMCs (13-16). However, a role of IGF-1 in HDAC activation has not been investigated. More importantly, despite the fact that IGF-1 receptor activation leads to a potent stimulation of mitogen activated protein kinases (MAPK) and phosphatidyl-inositol-3-kinase (PI3K)/protein kinase B (PKB) pathways (17, 18), the involvement of these pathways in HDAC phosphorylation and nuclear export remains unexplored in VSMCs. Therefore, in the present studies, we examined the effect of IGF-1 on the phosphorylation and nuclear export of HDAC5 in VSMCs and identified the signaling pathways involved in this process.

## Materials and Methods

***Antibodies and reagents:***

Cell culture reagents were obtained from Invitrogen Corp. (Grand Island, NY, USA) and IGF-1 was purchased from Peprotech Inc. (Rocky Hill, NJ, USA). AG1024 (IGF-1R inhibitor), AG1478 (EGFR inhibitor), PD98059 (MEK1 inhibitor), SP600125 (JNK inhibitor), SB203580 (p38 inhibitor), UO126 (MEK1 and MEK2 inhibitor), wortmannin (PI3-K inhibitor), SC-66 (AKT inhibitor) and DPI (NAD(P)H oxidase inhibitor) were purchased from Calbiochem (San Diego, CA, USA). NAC (ROS scavenger) was obtained from Sigma-Aldrich (Oakville, ON, Canada). Apocynin (NAD(P)H oxidase inhibitor) was purchased from Cayman Chemical (Ann Arbor, MI, USA). Total (#SC154) and phosphorylated ERK1/2 (#SC16982-R) antibodies as well as total HDAC5 (#SC11419) and Nox4 (#SC21860) were purchased from Santa Cruz Biotechnology (Dallas, TX, USA). β-tubulin (#2146S), total PKB (#9272S), phosphorylated PKB (#3787S), phosphorylated p38 MAPK (#4511S), phosphorylated JNK (#9251S), Lamin B1 (#12586) and anti-rabbit (#7074S) antibodies were obtained from Cell Signaling (Beverly, MA, USA). Phosphorylated HDAC5 (Serine 498) (#AB47283) was purchased from Abcam Inc (Toronto, ON, Canada). Alexa Fluor 488 goat anti-rabbit IgG (#A-11034) was obtained from Life Technologies, Molecular Probes (Grand Island, NY, USA). The enhanced chemiluminescence (ECL) detection system kit was from Perkin Elmer (Montreal, QC, Canada). Lipofectamine RNAi max (#13778-075) was provided by Life Technologies (Burlington, ON, Canada); SCR (#SR30004), PKB (#SR500756), and Nox4 (#SR506919) siRNA duplexes were obtained from Origene (Rockville, MD, USA).

***Cell culture:***

A10 VSMCs derived from the thoracic aorta of embryonic rats (CRL-1476 from ATCC (Manassas, VA, USA)) were maintained in culture with Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin, in a humidified atmosphere of 5% CO2 exchange at 37°C. They were sub-cultured twice a week by harvesting them with trypsin/ ethylenediaminetetraacetic acid (EDTA). The cells were grown to 80-90% confluence in 60-mm culture plates and made quiescent by incubating in FBS-free DMEM 5h prior to experimental treatment.

***Cell lysis and immunoblotting:***

Confluent serum-starved A10 VSMCs were incubated in the absence or presence of various reagents for 30 minutes, followed by stimulation with 50ng/ml IGF-1 (based on previous experiments performed in our lab, this was found to be the optimal concentration) for 5 min. The cells were washed three times with ice-cold PBS and lysed in 100 µL radio-immunoprecipitation (RIPA) buffer. Equal amounts of proteins measured by Bradford assay were subjected to 10% SDS-polyacrylamide gel electrophoresis, transferred to Immobilion-P polyvinylidinedifluoride membranes (Millipore, USA) and incubated overnight with respective primary antibodies: pHDAC5 (1:1000), total HDAC5 (1:1000) phospho-ERK1/2 (1:2000), total ERK1/2 (1:4000), total PKB (1:2000) phospho-PKB (1:2000), phospho-JNK (1:2000), total JNK (1:2000) phospho-p38 (1:2000), total p38 (1:2000) lamin B1 (1:2000), Nox4 (1:500) and β-tubulin (1:5000). The antigen-antibody complex was detected by horseradish peroxidase-conjugated secondary antibody (1:4000), and protein bands were visualized by ECL. The intensity of the bands was quantified by densitometric analysis using Quantity One Bio-Rad Corp. imaging and Graphpad Prism 5 (San Diego, CA, USA) software programs.

***Transfection with siRNA:***

Silencing of PKB or Nox4 was achieved by transfecting the VSMCs with specific small interfering RNAs as described earlier (19). VSMCs were transfected with siRNA duplexes against PKB or Nox4 or with non-specific duplexes (scrambled) according to the manufacturer's protocol. A mixture of the desired siRNA (PKB or Nox4) with lipofectamine or a mixture of scrambled siRNA with lipofectamine was added to the cells for a final concentration of 10 nM siRNA. The cells were incubated for 48h at 37$^{o}$C prior to treatment with IGF-1 or other agents.

***Immunofluorescence:***

Serum-starved A10 cells grown and treated on glass coverslips were washed with iced cold PBS and fixed with paraformaldehyde 4% for 30 min at 4°C. Permeabilization was achieved by 10 minutes incubation with 0.1% Triton X-100, 0.1% serum citrate pH 4.0 at room temperature (RT). Cells were then blocked with goat serum diluted in PBS (15 µL/mL PBS) for 1 hour and incubated overnight at 4°C with phospho-HDAC5 antibody diluted in the same blocking solution (1:100). Coverslips were further incubated for 2 hours at RT with Alexa Fluor 488 goat anti-rabbit IgG (1:150) Nuclei were then labelled by staining the coverslips with DAPI (2 µL/1.5 mL $H_2O$) before mounting them with a buffer made of 30% Glycerol in PBS. The images were taken using X-Cite Serie 120, TE2000-S fluorescence microscope (17).

***Nuclear extraction protocol:***

To separate the nuclear and cytosolic protein fractions, cells incubated in the absence or presence of pharmacological agents were lysed and nuclear and cytosolic protein were isolated for subsequent immunoblotting. Briefly, cells were washed three times with ice-cold PBS and collected in a 100µl buffer solution containing 10mM Hepes, 10mM KCl, 0.1mM EDTA, 0.1mM EGTA, 1mM PMSF, 1mM protease cocktail inhibitor and 1mM NaOV. Lysates were put on ice for 15 minutes before the addition of 10% NP40 detergent. Lysates were then vortexed for 10 seconds at highest setting before centrifugation at 18,327xg for 4 minutes at 4$^{o}$C. The supernatant (corresponding to the cytoplasmic fraction) was saved and transferred in a clean tube. The pellet was resuspended in 60µl, by pipeting up and down several times, in buffer containing 10mM Hepes, 400mM NaCl, 0.1mM EDTA, 0.1mM EGTA, 1mM PMSF, 1mM protease cocktail inhibitor and 1mM NaOV. Lysates were sonicated by performing 6 cycles at 10 seconds per cycle with 30 second intervals and then centrifuged at 18,327xg for 5 minutes at 4$^{o}$C. Pellet was discarded and the supernatant, corresponding to the nuclear fraction, was collected. Protein concentrations were measured using Bradford assay (17).

***Statistics:***

Statistical analysis was performed by one-way, standard analysis of variance (ANOVA). Significance of the differences between samples was assessed by a Tukey *post hoc* test. All data are reported as means ± SE of at least three different experiments. The differences between means were considered significant at $p< 0.05$.

## Results

### IGF-1 induces HDAC5 phosphorylation in a dose and time-dependent fashion through the IGF-1R in A10 VSMCs

Despite the fact that IGF-1 is a potent mitogen and is implicated in vascular dysfunction, its ability to induce phosphorylation of HDAC5 in VSMCs has not been explored. Therefore, we examined the effect of IGF-1 on HDAC5 phosphorylation in A10 aortic VSMCs. As depicted in Fig. 1A, treatment of VSMCs with IGF-1 increased HDAC5 phosphorylation at Ser498 in a dose-dependent manner, with 10ng/mL being the minimum required dose. Further increasing the IGF-1 concentration maintained the same level of HDAC5 phosphorylation. In addition, immunoblotting and immunofluorescence studies revealed that IGF-1-induced HDAC5 phosphorylation was very rapid, with a peak at 5 min and then a quick decline thereafter (Figs 1B and 1C). In order to confirm the involvement of IGF-1R in IGF-1-induced HDAC5 activation, we used AG1024, a highly specific inhibitor of IGF-1R tyrosine kinase activity. As depicted in Fig. 2, the use of AG1024 potently inhibited IGF-1-induced HDAC5 phosphorylation compared to cells treated with IGF-1 alone. Additionally, the use of AG1478, an inhibitor of EGFR tyrosine kinase activity, did not inhibit IGF-1-induced HDAC5 phosphorylation, further demonstrating the specific involvement of IGF-1R in this process (Fig. 2).

### IGF-1-induced HDAC5 phosphorylation is not MAPK pathway dependent

IGF-1 has been show to mediate its pathophysiological effects through the activation of hypertrophic and growth promoting signaling pathways, including the mitogen-activated protein

kinase (MAPK) pathway (17, 18). Therefore, we next investigated whether the MAPK pathway was involved in mediating IGF-1-induced HDAC5 phosphorylation. To investigate this and to identify which MAPKs are specifically involved in IGF-1-induced HDAC5 activation, a variety of pharmacological inhibitors were used. These included PD98059 and UO126, MEK1/2 inhibitors, as well as SP600125, a JNK inhibitor, and SB203580, a p38 MAPK inhibitor. As shown in Fig. 3, HDAC5 phosphorylation was strongly enhanced by IGF-1 and none of the MAPK inhibitors used attenuated this effect. These data indicated that the MAPK pathways are not involved in IGF-1-induced HDAC5 phosphorylation in A10 VSMCs.

**Activation of the PI3-K/PKB pathway is required for IGF-1-induced HDAC5 phosphorylation in A10 VSMCs**

Activation of the PI3-K/PKB pathway is critical to induce the cellular responses of IGF-1 (18). Therefore, to investigate whether the PI3-K/PKB pathway contributes to IGF-1-induced HDAC5 phosphorylation, we used wortmannin and SC-66, which are specific pharmacological inhibitors of PI3-K and PKB respectively. As shown in Fig. 4A, wortmannin treatment caused a significant reduction in HDAC5 phosphorylation in response to IGF-1. Similarly, pre-treatment of A10 VSMCs with SC-66 also suppressed IGF-1-induced phosphorylation of HDAC5 (Fig. 4B). Additionally, as shown in Fig. 4C, immunofluorescence experiments also confirmed that SC-66-induced inhibition of PKB reduced HDAC5 phosphorylation stimulated by IGF-1. We further probed the involvement of PKB in IGF-1-induced phosphorylation of HDAC5 by siRNA-mediated silencing of PKB in VSMCs. As shown in Fig 5A, siRNA-induced reduction in PKB levels resulted in suppression of HDAC5 phosphorylation in response to IGF-1, as revealed by

immunoblotting. Similar to immunoblotting, immunofluorescence microscopy also showed that siRNA-induced knockdown of PKB reduced the phosphorylation of HDAC5 (Fig. 5B). These data confirm the involvement of the PI3-K/PKB pathway in IGF-1-induced HDAC5 phosphorylation in VSMCs.

**IGF-1 triggers HDAC5 nuclear export in a PKB-dependent fashion in A10 VSMCs**

HDAC5 phosphorylation has been shown to lead to 14-3-3 chaperone protein binding and the subsequent nuclear export of phosphorylated HDAC5, resulting in the derepression of HDAC5 target genes (13). Phosphorylation of HDAC5 by either CaMKII or PKD has been shown to induce its nuclear export (13, 16). Therefore, we investigated whether IGF-1-induced phosphorylation of HDAC5 was associated with its nuclear export. As shown in Fig. 6, in unstimulated cells, HDAC5 is mainly localized in the nuclear fraction; however, treatment of the cells with IGF-1 resulted in a decrease in the levels of HDAC5 in the nuclear fraction and a concomitant increase in the cytosolic fraction. Moreover, treatment with SC-66, an inhibitor of PKB, disrupted the nuclear export of HDAC5, suggesting that IGF-1 stimulates HDAC5 nuclear export in a PKB-dependent manner in A10 VSMCs.

**ROS generation is required for IGF-1-induced HDAC5 phosphorylation and nuclear export in A10 VSMCs**

ROS play a significant role in the pathogenesis of vascular abnormalities (17). IGF-1 has been reported to mediate many of its effects through the activation of NAD(P)H oxidase-derived ROS generation (20). However, a role of ROS in IGF-1-induced HDAC5 phosphorylation in

VSMCs remains unexplored; therefore, with the use of diphenyleneiodonium (DPI) and apocynin, two NAD(P)H oxidase inhibitors, as well as *N*-acetylcysteine (NAC), a ROS scavenger, we investigated whether ROS is involved in IGF-1-induced HDAC5 phosphorylation. As depicted in Fig. 7, pre-treatment of VSMCs with DPI, NAC and apocynin resulted in a marked reduction in HDAC5 and PKB phosphorylation in response to IGF-1. Additionally, since Nox4 has been shown to be the main Nox isoform involved in mediating IGF-1's effects in VSMCs (21), we probed the involvement of Nox4 in IGF-1-induced phosphorylation of HDAC5 by siRNA-mediated silencing of Nox4 in VSMCs. As shown in Fig 8, siRNA-induced reduction in Nox4 levels resulted in suppression of HDAC5 and PKB phosphorylation in response to IGF-1, as revealed by immunoblotting. Furthermore, to investigate whether ROS generation triggers IGF-1-induced HDAC5 nuclear export, we treated cells with DPI and found that it disrupted the nuclear export of HDAC5, as indicated by high expression of HDAC5 in the nuclear fraction (Fig 9). These data suggest the involvement of Nox4-derived ROS generation in IGF-1-induced HDAC5 phosphorylation and nuclear export in A10 VSMCs.

## Discussion

In this study, we have demonstrated that IGF-1 stimulates the phosphorylation of HDAC5 at Serine 498 (Ser498) in A10 VSMCs and have identified the intracellular signaling pathways involved in this process. Our data reveal that pharmacological blockers of PI3-K and PKB, wortmannin and SC-66 respectively, attenuated IGF-1-induced phosphorylation of HDAC5, suggesting that PKB phosphorylates Ser498 on HDAC5 in response to IGF-1 in VSMCs. Furthermore, our results demonstrating that siRNA-induced silencing of PKB suppressed HDAC5 phosphorylation in response to IGF-1 provided additional support for a role of PKB in mediating the phosphorylation of HDAC5 in VSMC stimulated by IGF-1.

IGF-1-induced phosphorylation and activation of MAP kinases is among the first events triggered by IGF-1 receptor activation and has been shown to mediate several physiological responses of IGF-1 (18, 22). However, with the use of a series of pharmacological blockers of ERK1/2, JNK and p38 MAPK, we showed that none of these inhibitors reduced IGF-1-induced HDAC5 phosphorylation, suggesting that the MAPK pathway does not play a role in mediating IGF-1-induced HDAC5 phosphorylation in VSMCs.

Earlier studies have reported that angiotensin II (AngII), platelet derived growth factor (PDGF) and Ca2+ elevating agents induce phosphorylation of HDAC5 at Ser259 and Ser498 in VSMCs and in intestinal epithelial cells (13, 15, 16, 21). These studies also identified protein

kinase D1(PKD1) (16, 21), which is activated in a protein kinase C (PKC)-dependent manner, and calcium/calmodulin-dependent protein kinase II (CaMKII) (13) as the putative protein kinases responsible for catalyzing the phosphorylation of these serine residues in HDAC5. AngII- and PDGF-induced HDAC5 phosphorylation have been suggested to play a role in VSMC proliferation and hypertrophy, and modulate the transcriptional activity of cell cycle regulatory proteins (13, 15, 16). However, our studies provide first evidence for a role of PKB in mediating IGF-1-induced HDAC5 phosphorylation at Ser498 in VSMCs.

A role of ROS generated by NAD(P)H oxidase 4 (Nox4) in mediating IGF-1 signaling, as well as IGF-1's proliferative and migratory effects has been previously reported (20). We have also shown earlier that IGF-1-induced phosphorylation of PKB and enhanced Egr-1 expression are mediated by a ROS-dependent pathway (17). Our observation that DPI and apocynin, two pharmacological blockers of NAD(P)H oxidase, as well as the ROS scavenger NAC significantly reduced both HDAC5 phosphorylation and PKB phosphorylation in response to IGF-1 in VSMCs indicated the involvement of ROS in IGF-1-induced HDAC5 phosphorylation in VSMCs. Furthermore, our results showing that silencing of Nox4 by siRNA potently reduced both HDAC5 and PKB phosphorylation in response to IGF-1 in VSMCs, suggested that Nox4 activation is critical to enhance ROS generation in response to IGF-1 in VSMCs.

HDAC5 phosphorylation has been shown to result in its exclusion from the nucleus in cells treated with AngII, PDGF, and other agents (13, 16). Using nuclear and cytoplasmic fractionation techniques, we have also provided evidence that, similar to AngII and PDGF, IGF-

1 was also able to induce the nuclear export of HDAC5 in a PKB- and ROS-dependent fashion in VSMCs. To the best of our knowledge, the work presented here has revealed a previously unidentified role of PI3-K/PKB signaling pathway in inducing the phosphorylation and nuclear export of HDAC5 in response to IGF-1 in VSMCs.

In summary, our data demonstrate the involvement of the PI3-K/PKB pathway as well as Nox4 as upstream regulators of HDAC5 phosphorylation and nuclear export in response to IGF-1. Since HDAC phosphorylation has been associated with enhanced migration and proliferation of VSMCs by altered expression of genes associated with cell cycle regulation, it may be suggested that PKB- and Nox4-generated ROS-induced phosphorylation of HDAC5 in VSMCs may constitute a potential mechanism to trigger a signaling program leading to vascular remodelling.

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## Disclosures

The authors of this manuscript do not have any conflict of interest to disclose.

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**Figure 1. IGF-1 stimulates HDAC5 phosphorylation in A10 VSMCs in a time and dose-dependent fashion**

Confluent serum-starved quiescent A10 cells were incubated with increasing concentrations of IGF-1 for 5 minutes (A) or with 50ng/ml IGF-1 for the indicated time periods (B). Cell lysates were probed with pHDAC5 antibody (Ser498), as shown in the top panels of each section (A and B). Cell lysates were also probed with pPKB and also analyzed for protein loading using total HDAC5. Bar diagrams represent the average quantification of HDAC5 phosphorylation relative to total HDAC5. Values are the means ± SE of 3 independent experiments and expressed as a ratio where the control values are taken as 1. ***P<0.0005 compared to CTL. (C) shows immunofluorescence microscopy of pHDAC5 cellular localization in IGF-1 treated VSMCs. Cells growing on coverslips were fixed and stained with anti-phospho-HDAC5 antibody (green signal). Nuclei were stained with DAPI (4′,6-diamidino-2-phenylindole) (blue signal). Merged pictures show the DAPI-stained image superimposed on the pHDAC5-stained image. Shown are representative images from 3 independent experiments. The images were taken using X-Cite series 120, TE2000-S fluorescence microscope.

**Figure 2. IGF-1 – induced HDAC5 phosphorylation is attenuated by AG1024 (IGF-1R-PTK inhibitor) and not by AG1478 (EGFR TK inhibitor) in A10 VSMCs**

Confluent serum-starved quiescent A10 VSMCs were pre-treated without (-) or with (10μM) AG1024 (IGF-1R-PTK inhibitor) or without (-) or with (10μM) AG1478 (EGFR-PTK inhibitor) for 30 minutes, followed by stimulation with 50ng/ml IGF-1 for 5 minutes. Cell lysates were probed with pHDAC5 antibody, with pPKB and also analyzed for protein loading using a total HDAC5 antibody. Bar diagrams represent the average quantification of HDAC5 phosphorylation

relative to total HDAC5. Values are the means ± SE of 3 independent experiments expressed as a ratio where the control values are taken as 1. *** P<0.0005 compared with the control VSMC and ### P<0.0005 to VSMC treated with IGF-1 alone.

**Figure 3. Pharmacological inhibitors of MAP Kinases (PD98059 (MEK1/2 inhibitor), UO126 (MEK1/2 inhibitor), SP600125 (JNK inhibitor) and SB203580 (p38 MAPK inhibitor)) did not inhibit IGF-1-induced HDAC5 phosphorylation in A10 VSMCs**

Confluent, serum-starved quiescent A10 VSMCs were pre-treated without (-) or with (10uM) PD98059 (MEK1/2 inhibitor) (A), UO126 (MEK1/2 inhibitor) (B), SP600125 (JNK inhibitor) (C) or SB203580 (p38 MAPK inhibitor) (D) for 30 minutes, followed by stimulation with 50ng/ml IGF-1 for 5 minutes. Cell lysates were probed with pHDAC5 antibody, probed with either phospho-ERK1/2, phospho-p38 or phospho-JNK and also analyzed for protein loading using a total HDAC5 antibody. Bar diagrams represent the average quantification of HDAC5 phosphorylation relative to total HDAC5. Values are the means ± SE of 3 independent experiments and expressed as a ratio where the control values are taken as 1. *** P<0.0005 compared with the control.

**Figure 4. Attenuation of IGF-1-induced HDAC5 phosphorylation by pharmacological inhibitors wortmannin (PI3-K inhibitor) and SC-66 (PKB inhibitor) in A10 VSMCs**

Confluent, serum-starved quiescent A-10 VSMCs were pre-treated with either wortmannin (PI3-K inhibitor) (A) or SC-66 (PKB inhibitor) (B) for 30 minutes at indicated concentrations, followed by stimulation with 50ng/ml IGF-1 for 5 minutes. Cell lysates were probed with

pHDAC5 antibody, probed with pPKB and also analyzed for protein loading using a total HDAC5 antibody. Bar diagrams represent the average quantification of HDAC5 phosphorylation relative to total HDAC5. Values are the means ± SE of 3 independent experiments and expressed as a ratio where the control values are taken as 1. ***P<0.0005 compared with the control VSMC and ###P<0.0005 compared to VSMC treated with IGF-1 alone. C) shows immunofluorescence microscopy of pHDAC5 cellular localization in SC-66 and IGF-1 treated VSMCs. Cells growing on coverslips were fixed and stained with anti-phospho-HDAC5 antibody (green signal). Nuclei were stained with DAPI (4′,6-diamidino-2-phenylindole) (blue signal). Merged pictures show the DAPI-stained image superimposed on the pHDAC5-stained image. Shown are representative images from 3 independent experiments. The images were taken using X-Cite series 120, TE2000-S fluorescence microscope.

**Figure 5. siRNA-induced silencing of PKB inhibits IGF-1-induced HDAC5 phosphorylation in A10 VSMCs**

A10 VSMCs were transfected with 10nM PKB siRNA or 10 nM scrambled siRNA for 48 h prior to stimulation with 50ng/ml nM IGF-1 for 5 min. A) Cell lysates were immunoblotted with pHDAC5, with PKB total or using a total HDAC5 antibody. Bar diagrams represent the average quantification of HDAC5 phosphorylation relative to total HDAC5. Values are the means ± SE of at least 3 independent experiments and are expressed as fold increase compared to the control value (CTL). ***P<0.0005 is considered statistically significant versus CTL. B) shows immunofluorescence microscopy of pHDAC5 cellular localization in PKB-silenced and IGF-1

treated VSMCs. Cells growing on coverslips were fixed and stained with anti-phospho-HDAC5 antibody (green signal). Nuclei were stained with DAPI (4´,6-diamidino-2-phenylindole) (blue signal). Merged pictures show the DAPI-stained image superimposed on the pHDAC5-stained image. Shown are representative images from 3 independent experiments. The images were taken using X-Cite series 120, TE2000-S fluorescence microscope.

**Figure 6. Nuclear and cytoplasmic fractionation experiments illustrate that IGF-1-induced nuclear export of HDAC5 is mediated by PKB**

Confluent, serum-starved quiescent A-10 VSMCs were pre-treated with 10μM SC-66 for 30 minutes, followed by stimulation with 50ng/ml IGF-1 for 5 minutes. Cell lysates were probed with total HDAC5 antibody (top panel), with β-tubulin to control for the purity of the cytosolic fraction (middle panel) and also analyzed with lamin B1 to control for the purity of the nuclear fraction (bottom panel). Bar diagrams represent the average quantification of total HDAC5 relative to β-tubulin (cytosolic fraction) or total HDAC5 relative to lamin B1 (nuclear fraction) . Values are the means ± SE of 3 independent experiments and expressed as a ratio where the control values are taken as 1. ***P<0.0005 compared with the control VSMC and ###P<0.0005 compared to VSMC treated with IGF-1 alone.

**Figure 7. ROS inhibitors Diphenyleneiodonium (DPI), Apocynin and N-Acetyl-Cysteine (NAC) inhibited IGF-1-induced HDAC5 and PKB phosphorylation in A10 VSMCs**

Confluent, serum-starved quiescent A-10 VSMCs were pre-treated without (-) or with (10μM) DPI (A), without (-) or with (10μM) Apocynin (B) or without (-) or with (10μM) NAC (C) for 30 minutes, followed by stimulation with 50ng/ml IGF-1 for 5 minutes. Cell lysates were probed with pHDAC5 antibody, with pPKB antibody and were also analyzed for protein loading using a total HDAC5 antibody. Bar diagrams represent the average quantification of HDAC5 phosphorylation relative to total HDAC5. Values are the means ± SE of at 3 independent experiments and expressed as a ratio where the control values are taken as 1. *** $P<0.0005$, compared with the control VSMCs and ###$P<0.0005$ to VSMC treated with IGF-1 alone.

**Figure 8. siRNA-induced silencing of Nox4 inhibits IGF-1-induced HDAC5 phosphorylation in A10 VSMCs**

A10 VSMCs were transfected with 10nM Nox4 siRNA or 10 nM control siRNA for 48 h prior to stimulation with 50ng/ml nM IGF-1 for 5 min. Cell lysates were immunoblotted with pHDAC5, total HDAC5, pPKB, total PKB and Nox4 antibodies. Bar diagrams represent the average quantification of HDAC5 phosphorylation relative to total HDAC5 or the quantification of PKB phosphorylation relative to total PKB. Values are the means ± SE of at least 3 independent experiments and are expressed as fold increase compared to the control value (CTL). ***$P<0.0005$ is considered statistically significant versus CTL.

**Figure 9. Nuclear and cytoplasmic fractionation experiments illustrate that IGF-1-induced nuclear export of HDAC5 is ROS dependent**

Confluent, serum-starved quiescent A-10 VSMCs were pre-treated with 10μM DPI for 30 minutes, followed by stimulation with 50ng/ml IGF-1 for 5 minutes. Cell lysates were probed with total HDAC5 antibody (top panel), with β-tubulin to control for the purity of the cytosolic fraction (middle panel) and also analyzed with lamin B1 to control for the purity of the nuclear fraction (bottom panel). Bar diagrams represent the average quantification of total HDAC5 relative to β-tubulin (cytosolic fraction) or total HDAC5 relative to lamin B1 (nuclear fraction). Values are the means ± SE of 3 independent experiments and expressed as a ratio where the control values are taken as 1. ***P<0.0005 compared with the control VSMC and ###P<0.0005 compared to VSMC treated with IGF-1 alone.

**Figure 10: Schematic model of the involvement of PKB and ROS signaling in IGF-1-induced HDAC5 phosphorylation and nuclear export in A10 VSMC**

In VSMCs, IGF-1 functions as a high-affinity ligand for IGF-1R in the cellular membrane, leading to autophosphorylation of IGF-1R and recruitment of the IRS adaptor proteins. The interaction of IGF-1R with the IRS proteins induces the activation of the class I phosphatidyl inositol 3' kinase (PI3K). PI3K converts PIP2 into the lipid second messenger PIP3. PIP3 then activates PDK1 and PDK2, which go on to phosphorylate PKB at threonine 308 (Thr308) and serine 473 (Ser473), respectively. Activated PKB leads to the phosphorylation and export of HDAC5. Additionally, IGF-1R activation can lead to increased ROS generation via NAD(P)H oxidase activation, triggering the PKB and MAPK pathways. IGF-1-induced ROS generation increases IGF-1-induced HDAC5 phosphorylation and nuclear export. Contrastingly, activation of the MAPK pathway is not involved in IGF-1-induced HDAC5 phosphorylation.

Figure 1

A)

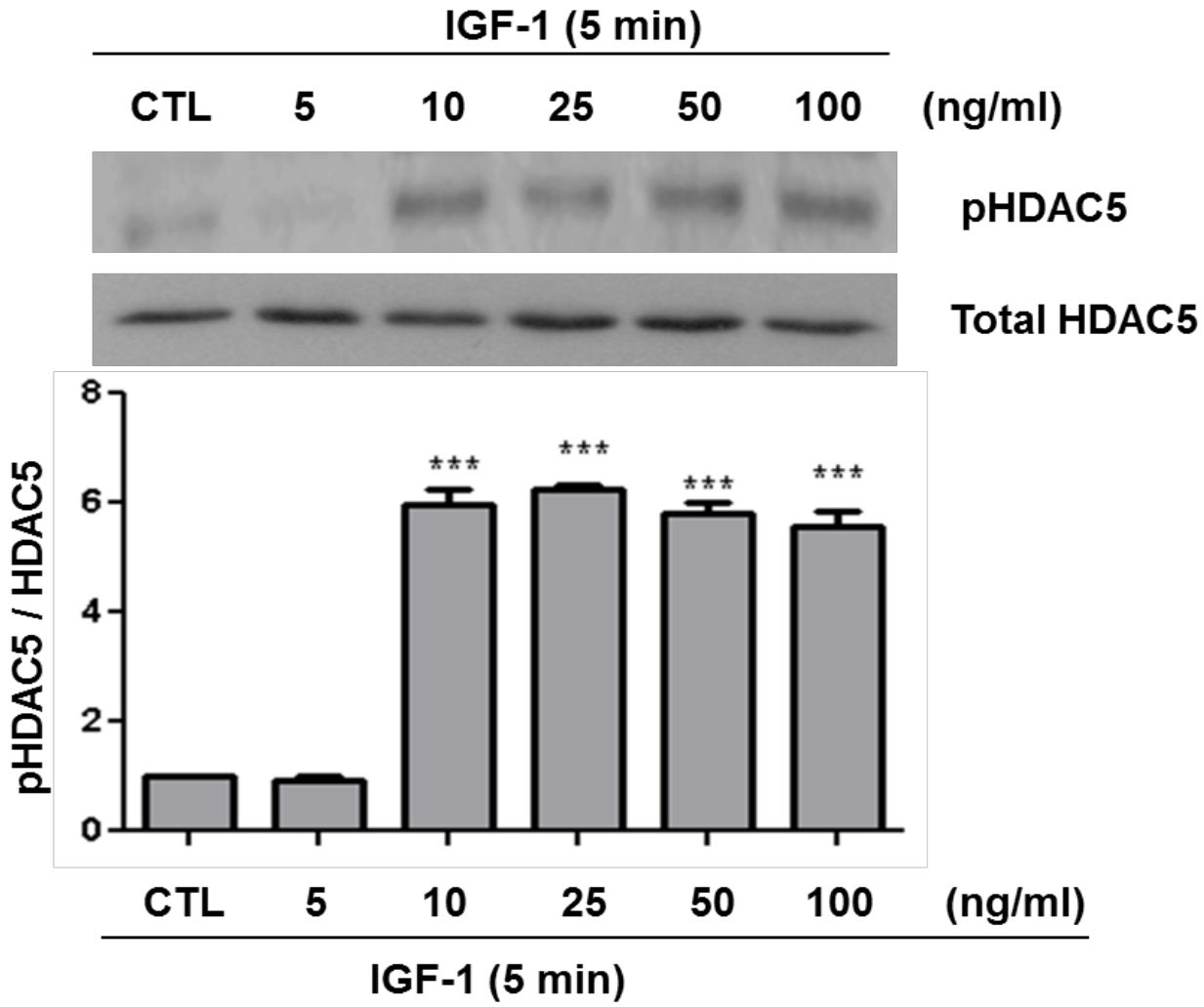

IGF-1 (5 min)
CTL
5
10
25
50
100
(ng/ml)
pHDAC5
Total HDAC5
pHDAC5 / HDAC5
0
2
4
6
8
***
***
***
***
CTL
5
10
25
50
100
(ng/ml)
IGF-1 (5 min)

Figure 1
B)

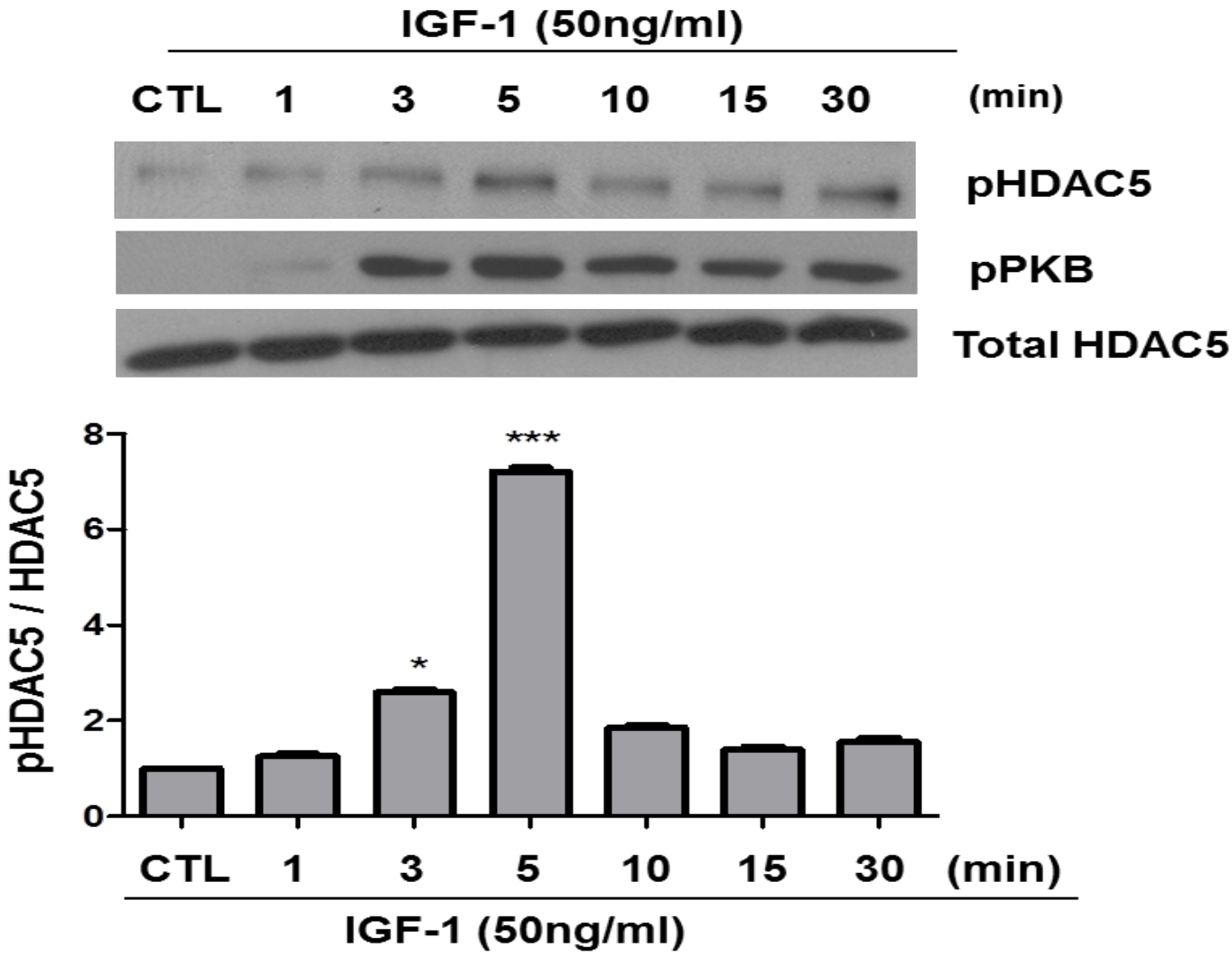

IGF-1 (50ng/ml)
CTL 1 3 5 10 15 30 (min)
pHDAC5
pPKB
Total HDAC5
pHDAC5 / HDAC5
8
6
4
2
0
*
***
CTL 1 3 5 10 15 30 (min)
IGF-1 (50ng/ml)

Figure 1
C)

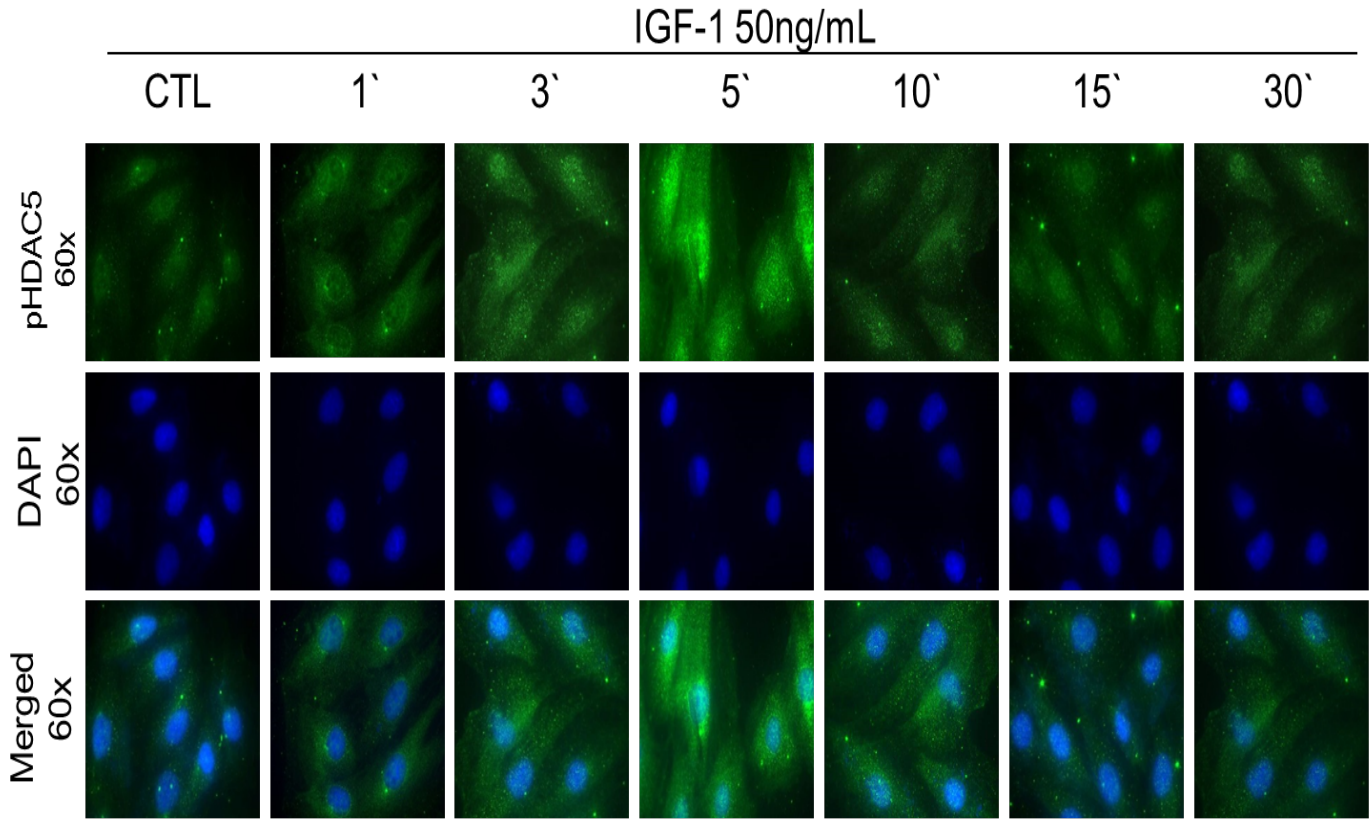


Figure 2

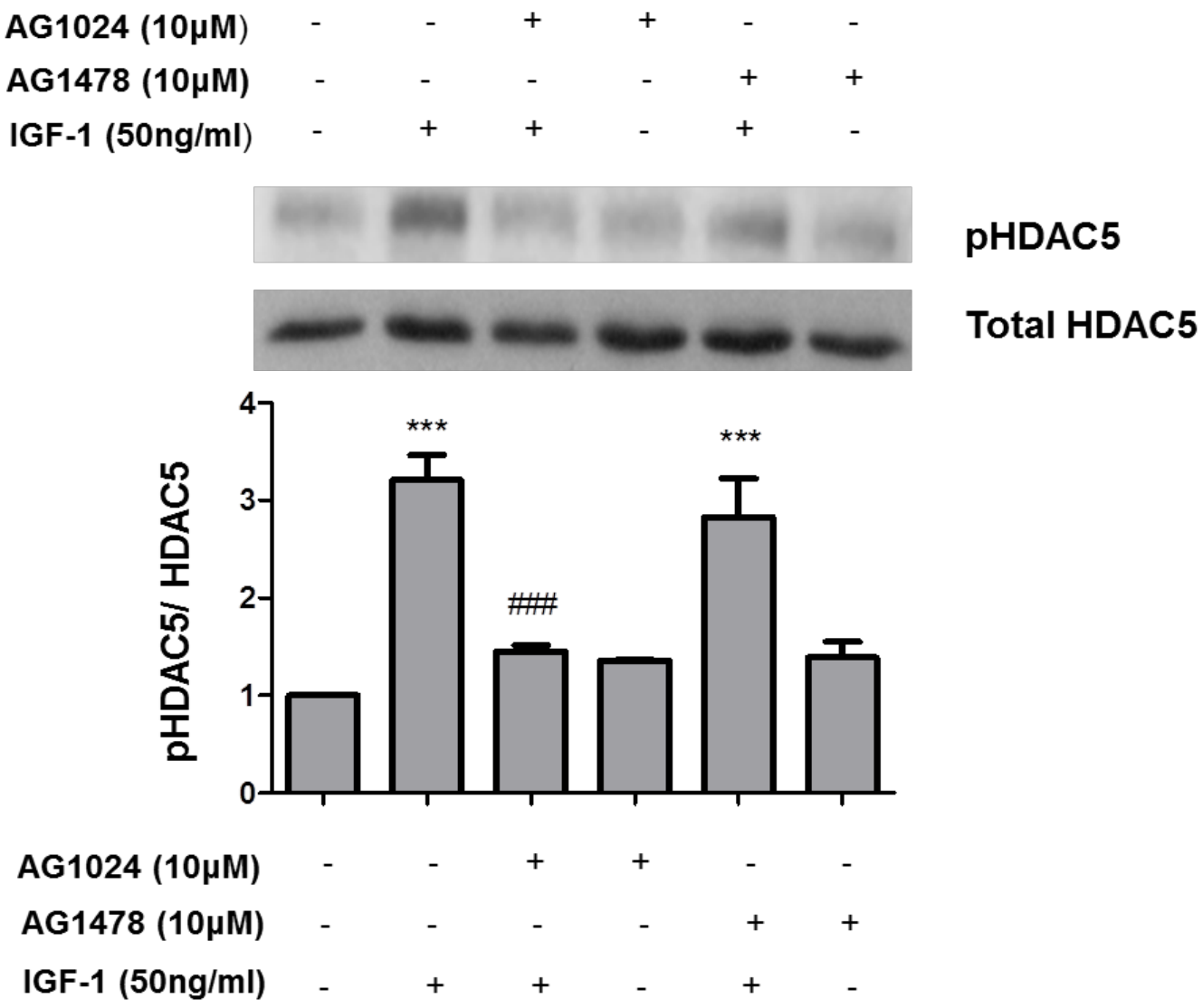

AG1024 (10μM) - - + + - -
AG1478 (10μM) - - - - + +
IGF-1 (50ng/ml) - + + - + -
pHDAC5
Total HDAC5
pHDAC5/ HDAC5
0
1
2
3
4
***
###
***
AG1024 (10μM) - - + + - -
AG1478 (10μM) - - - - + +
IGF-1 (50ng/ml) - + + - + -

Figure 3

A)

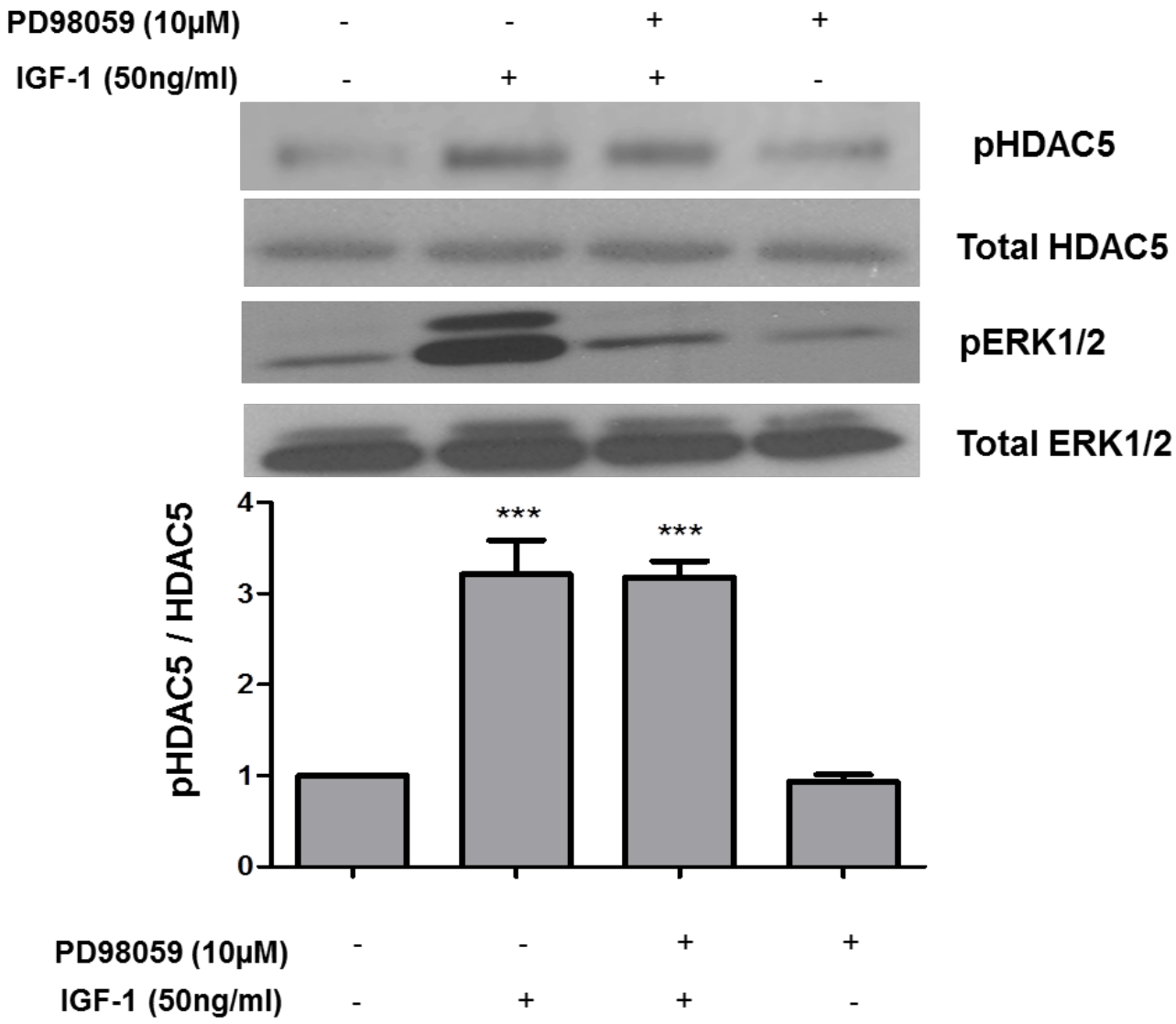

PD98059 (10μM) - - + +
IGF-1 (50ng/ml) - + + -
pHDAC5
Total HDAC5
pERK1/2
Total ERK1/2
pHDAC5 / HDAC5
0
1
2
3
4
***
***
PD98059 (10μM) - - + +
IGF-1 (50ng/ml) - + + -

Figure 3

B)

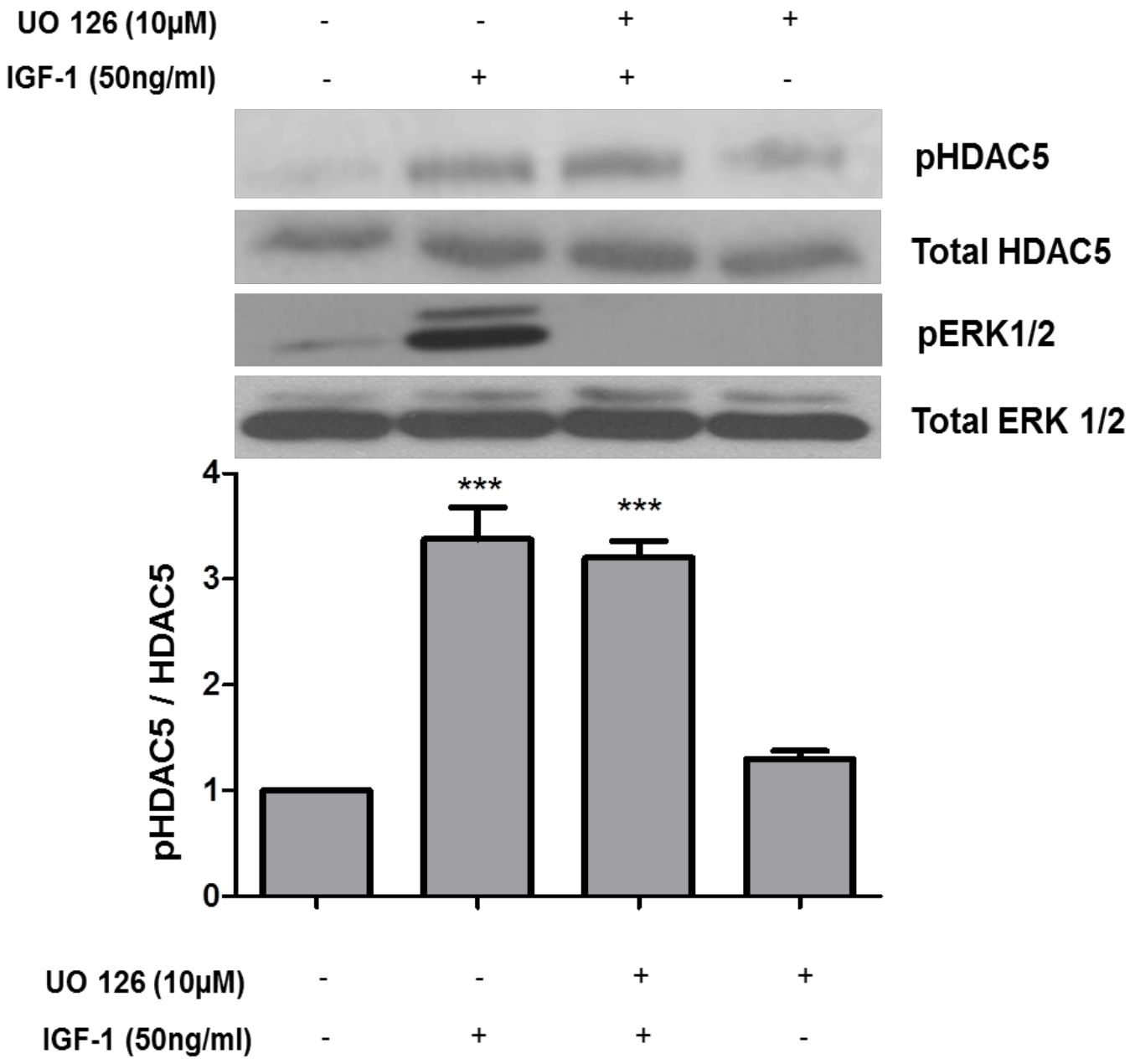

UO 126 (10μM) - - + +
IGF-1 (50ng/ml) - + + -
pHDAC5
Total HDAC5
pERK1/2
Total ERK 1/2
pHDAC5 / HDAC5
0
1
2
3
4
***
***
UO 126 (10μM) - - + +
IGF-1 (50ng/ml) - + + -

Figure 3

C)

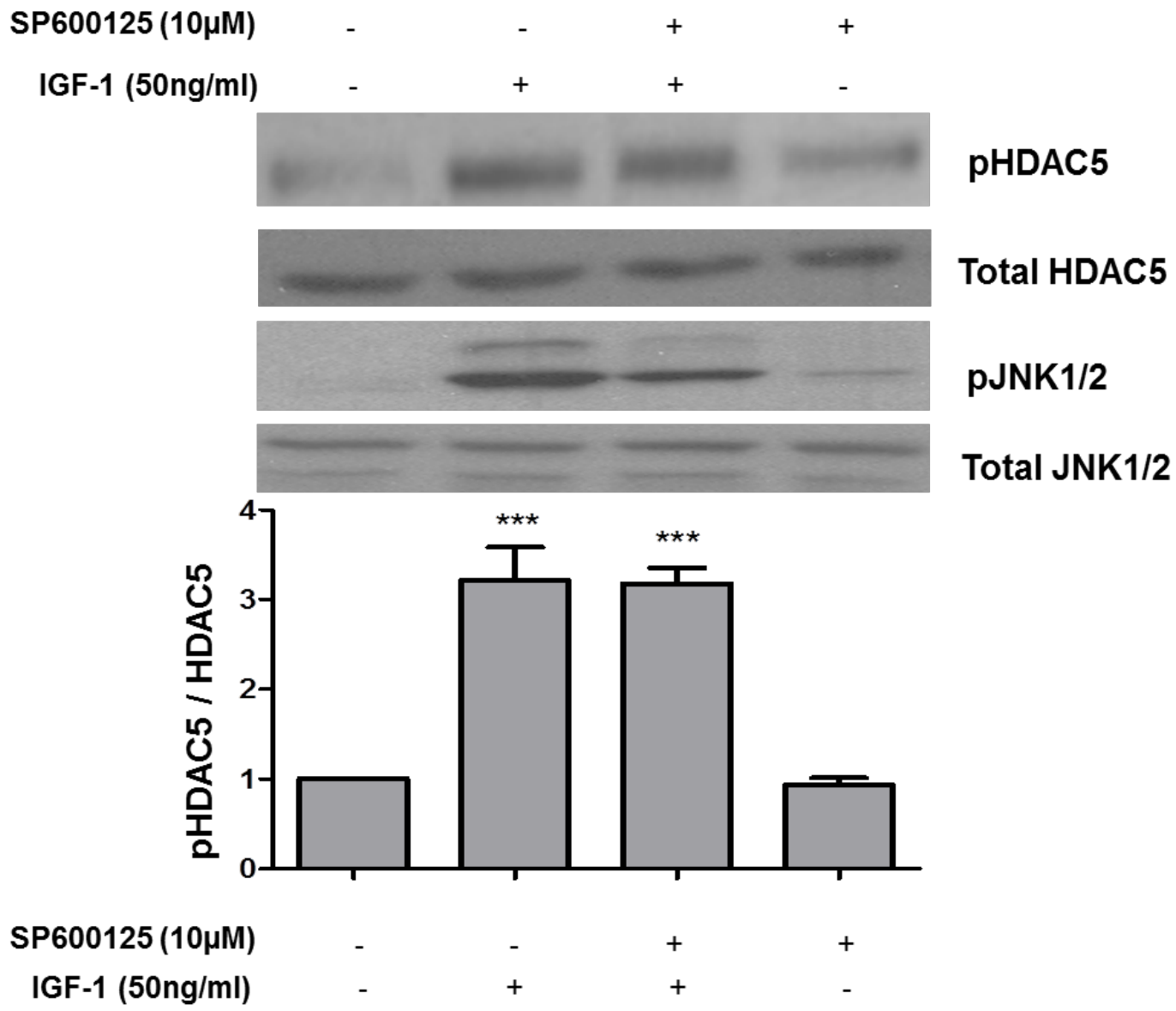

SP600125 (10μM) - - + +
IGF-1 (50ng/ml) - + + -
pHDAC5
Total HDAC5
pJNK1/2
Total JNK1/2
pHDAC5 / HDAC5
0
1
2
3
4
***
***
SP600125 (10μM) - - + +
IGF-1 (50ng/ml) - + + -

Figure 3

D)

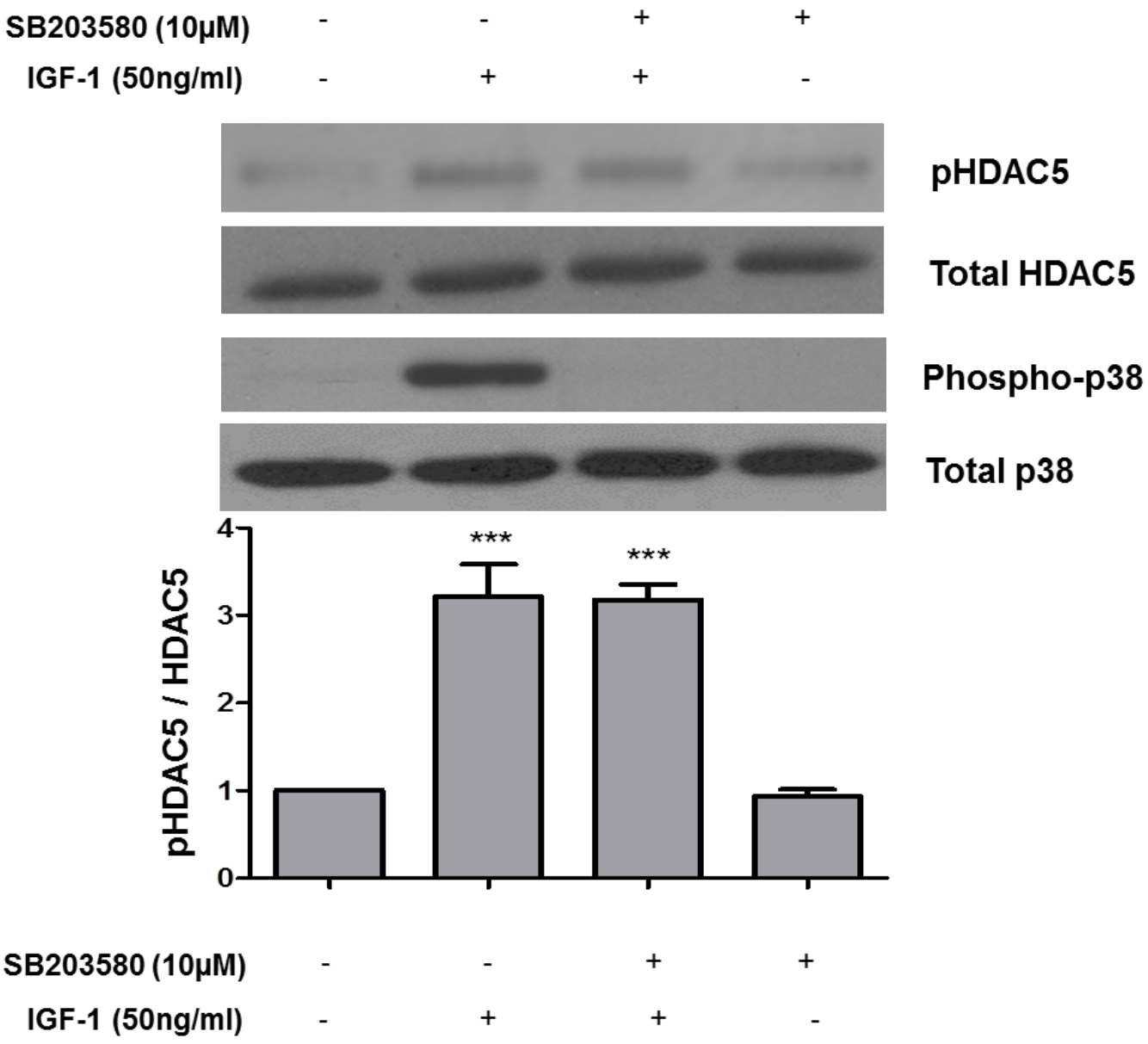

SB203580 (10μM) - - + +
IGF-1 (50ng/ml) - + + -
pHDAC5
Total HDAC5
Phospho-p38
Total p38
pHDAC5 / HDAC5
0
1
2
3
4
***
***
SB203580 (10μM) - - + +
IGF-1 (50ng/ml) - + + -

Figure 4

A)

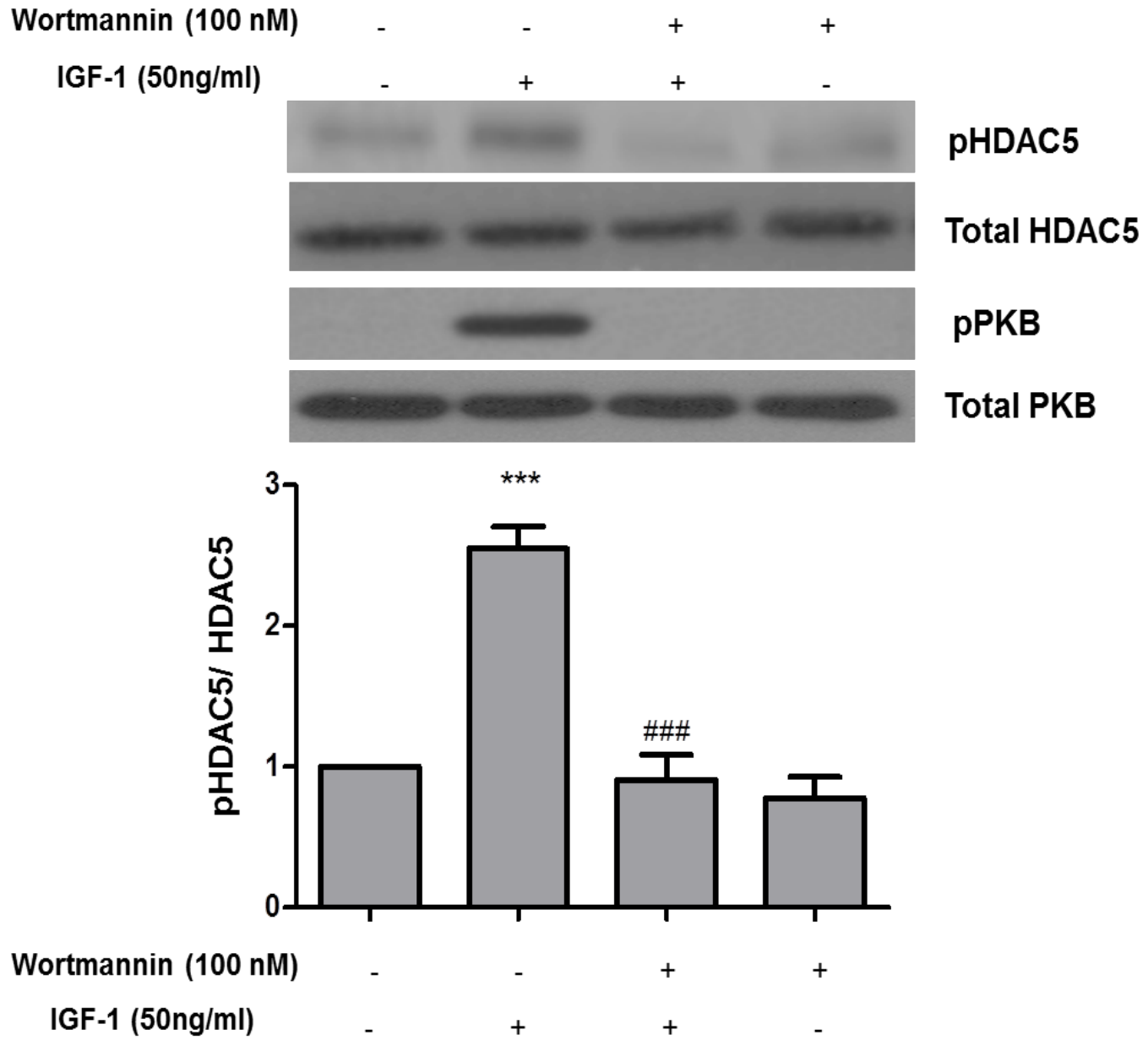

Wortmannin (100 nM) - - + +
IGF-1 (50ng/ml) - + + -
pHDAC5
Total HDAC5
pPKB
Total PKB
pHDAC5/ HDAC5
3
2
1
0
***
###
Wortmannin (100 nM) - - + +
IGF-1 (50ng/ml) - + + -

Figure 4

B)

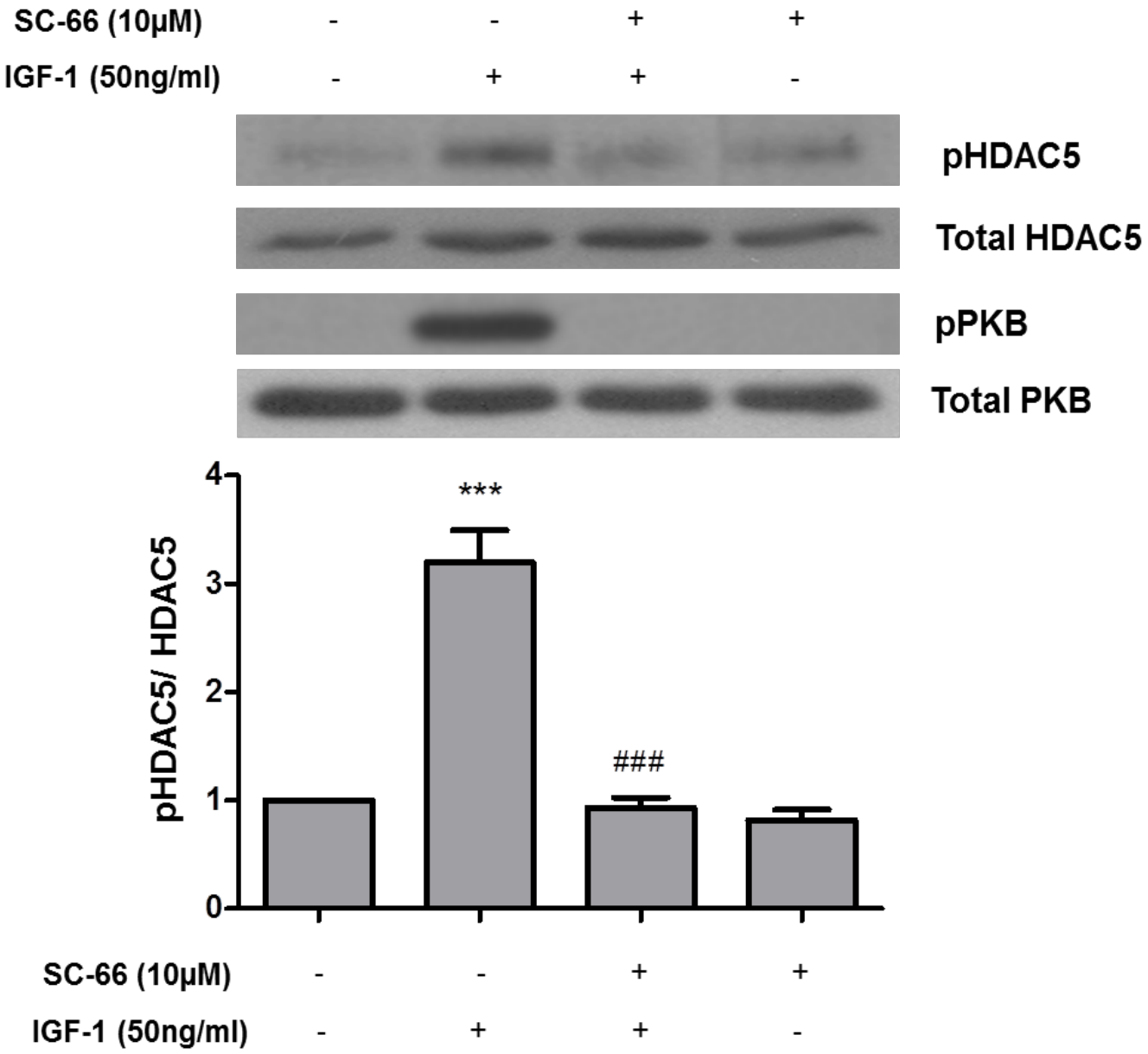

SC-66 (10μM) - - + +
IGF-1 (50ng/ml) - + + -
pHDAC5
Total HDAC5
pPKB
Total PKB
pHDAC5/ HDAC5
0
1
2
3
4
***
###
SC-66 (10μM) - - + +
IGF-1 (50ng/ml) - + + -

Figure 4

C)

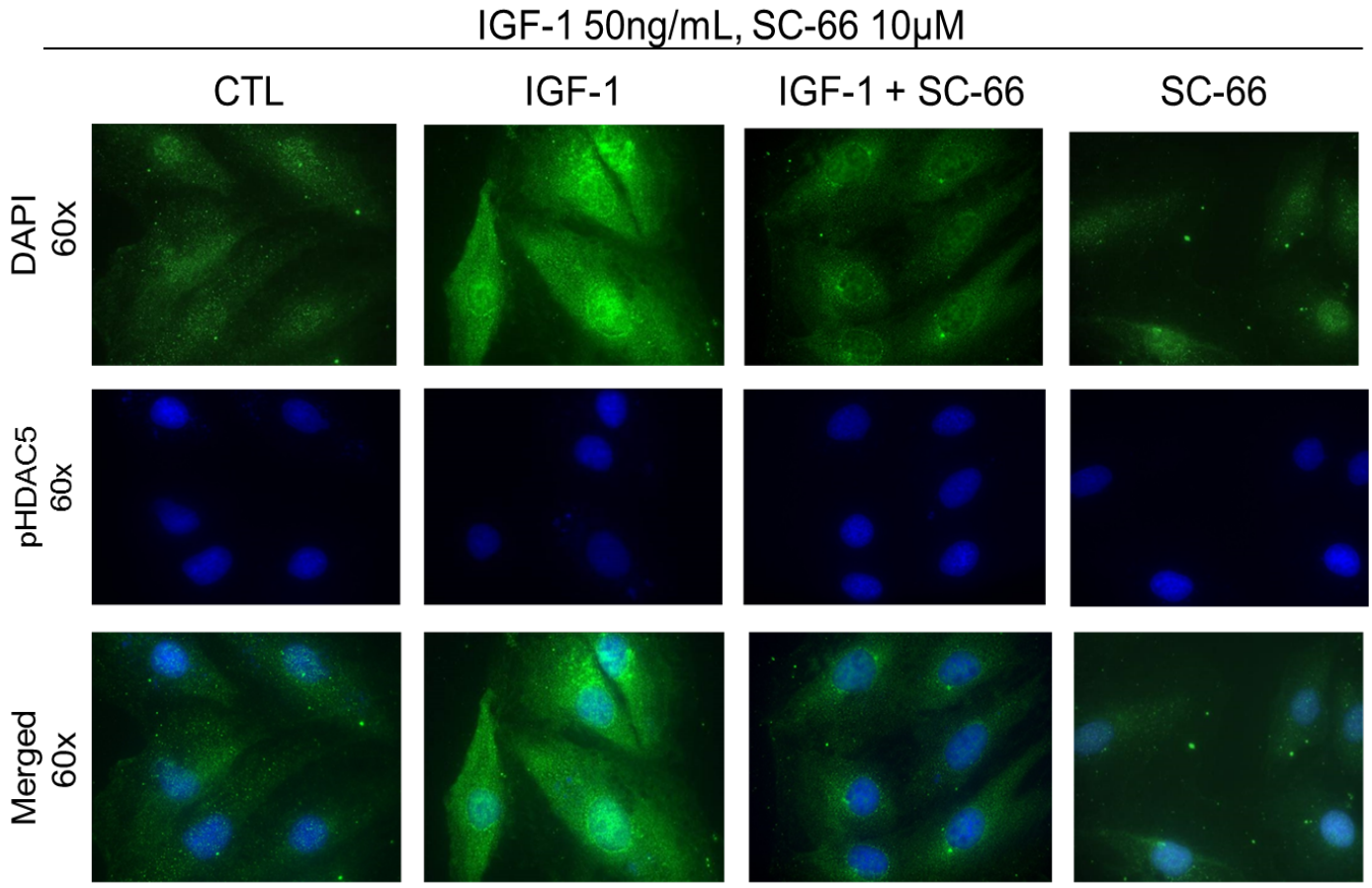

IGF-1 50ng/mL, SC-66 10μM
CTL
IGF-1
IGF-1 + SC-66
SC-66
DAPI 60x
pHDAC5 60x
Merged 60x

Figure 5

A)

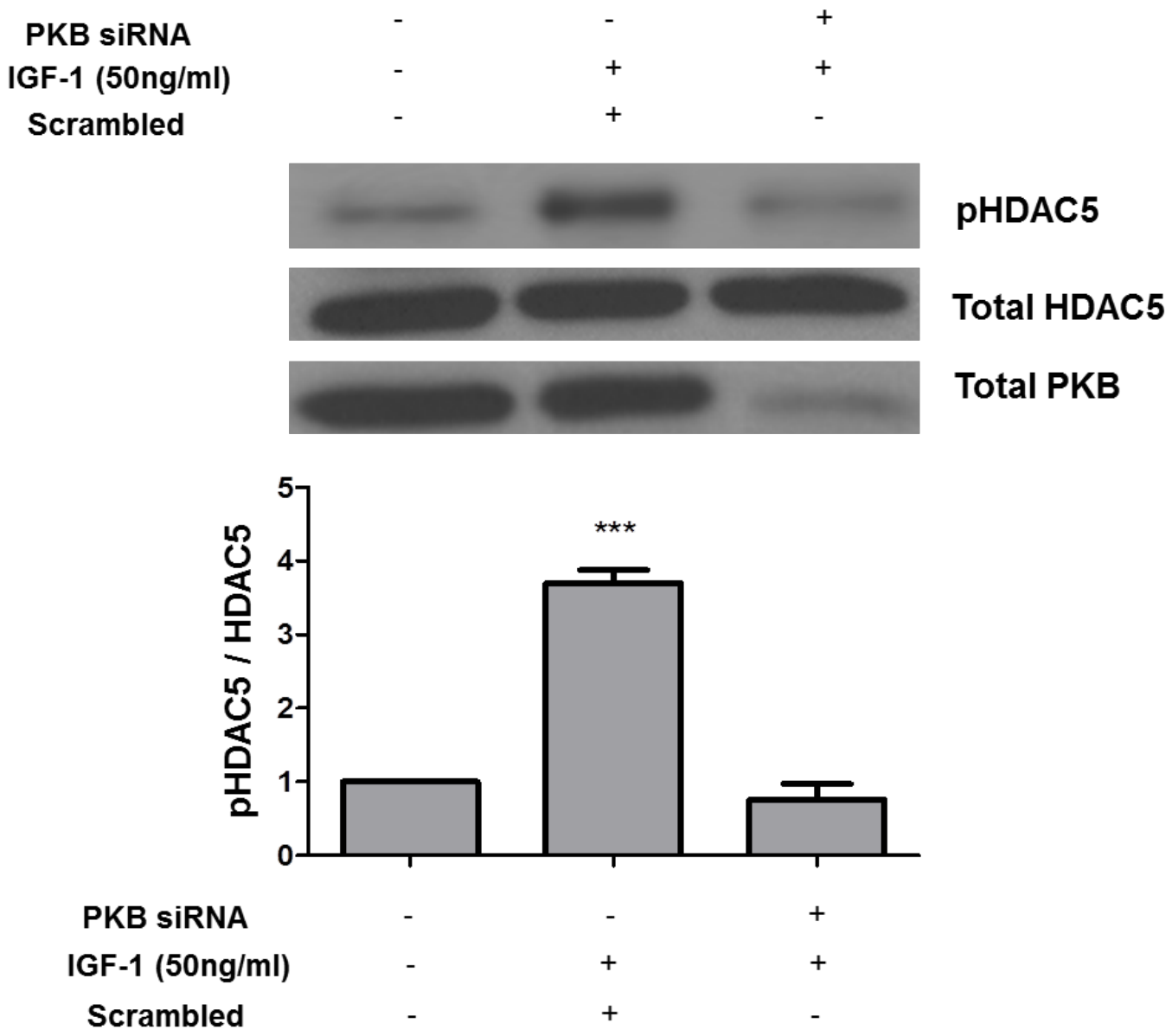

PKB siRNA - - +
IGF-1 (50ng/ml) - + +
Scrambled - + -
pHDAC5
Total HDAC5
Total PKB
pHDAC5 / HDAC5
0
1
2
3
4
5
***
PKB siRNA - - +
IGF-1 (50ng/ml) - + +
Scrambled - + -

Figure 5

B)

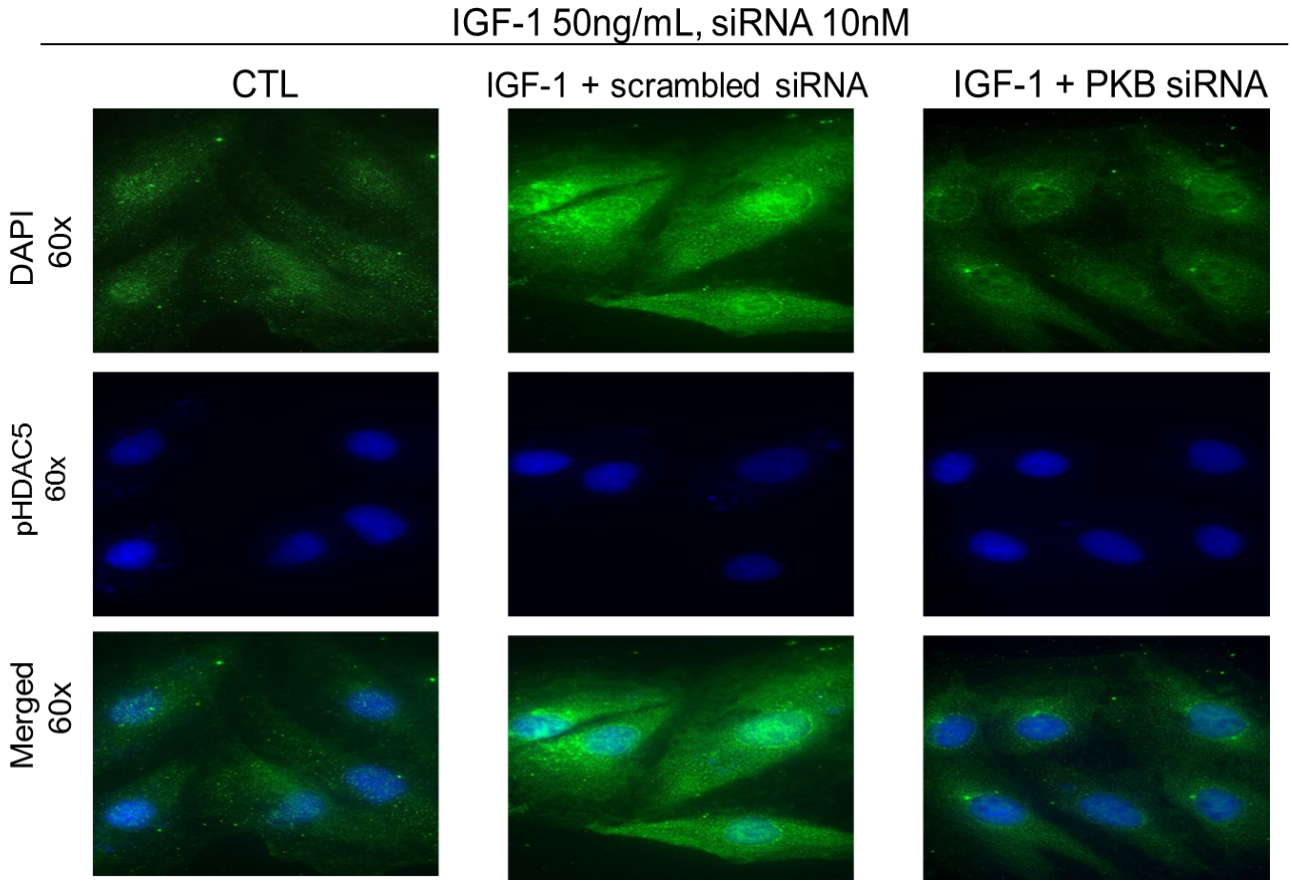

IGF-1 50ng/mL, siRNA 10nM
CTL
IGF-1 + scrambled siRNA
IGF-1 + PKB siRNA
DAPI 60x
pHDAC5 60x
Merged 60x

Figure 6

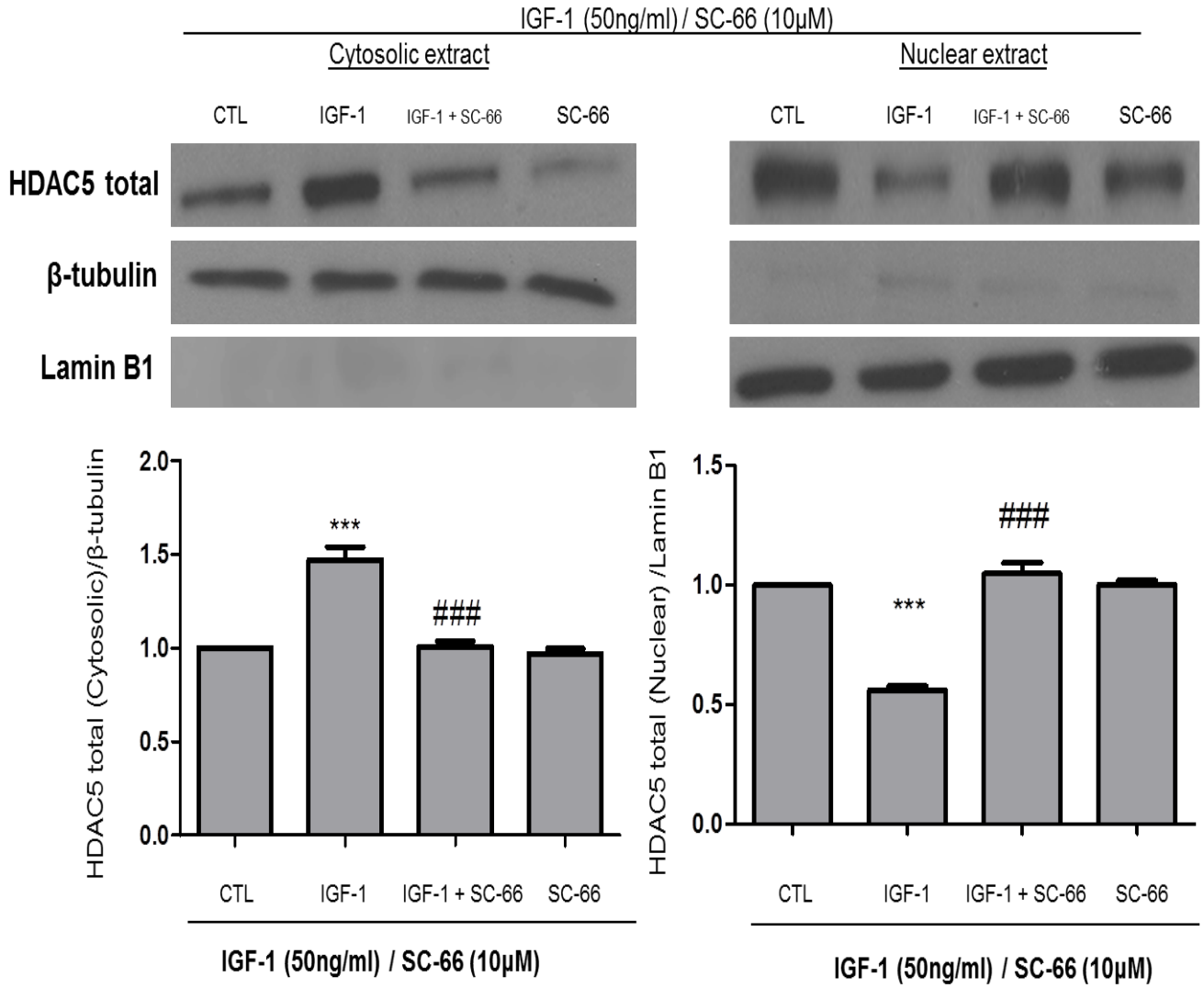

IGF-1 (50ng/ml) / SC-66 (10μM)
Cytosolic extract
Nuclear extract
CTL
IGF-1
IGF-1 + SC-66
SC-66
HDAC5 total
β-tubulin
Lamin B1
HDAC5 total (Cytosolic)/β-tubulin
2.0
1.5
1.0
0.5
0.0
***
###
HDAC5 total (Nuclear) /Lamin B1
IGF-1 (50ng/ml) / SC-66 (10μM)

Figure 7

A)

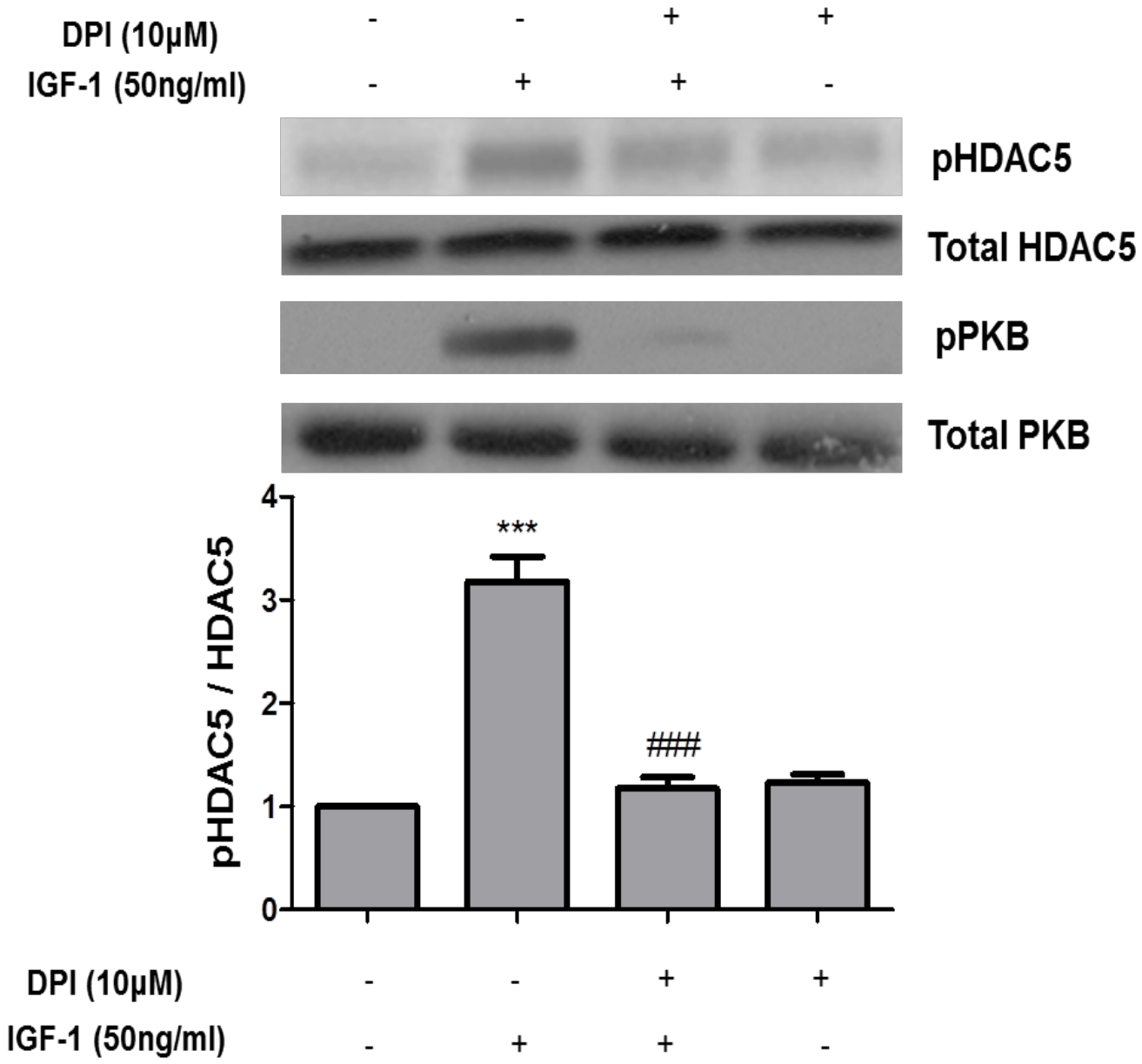

DPI (10μM)
- - + +
IGF-1 (50ng/ml)
- + + -
pHDAC5
Total HDAC5
pPKB
Total PKB
pHDAC5 / HDAC5
0
1
2
3
4
***
###
DPI (10μM)
- - + +
IGF-1 (50ng/ml)
- + + -

Figure 7

B)

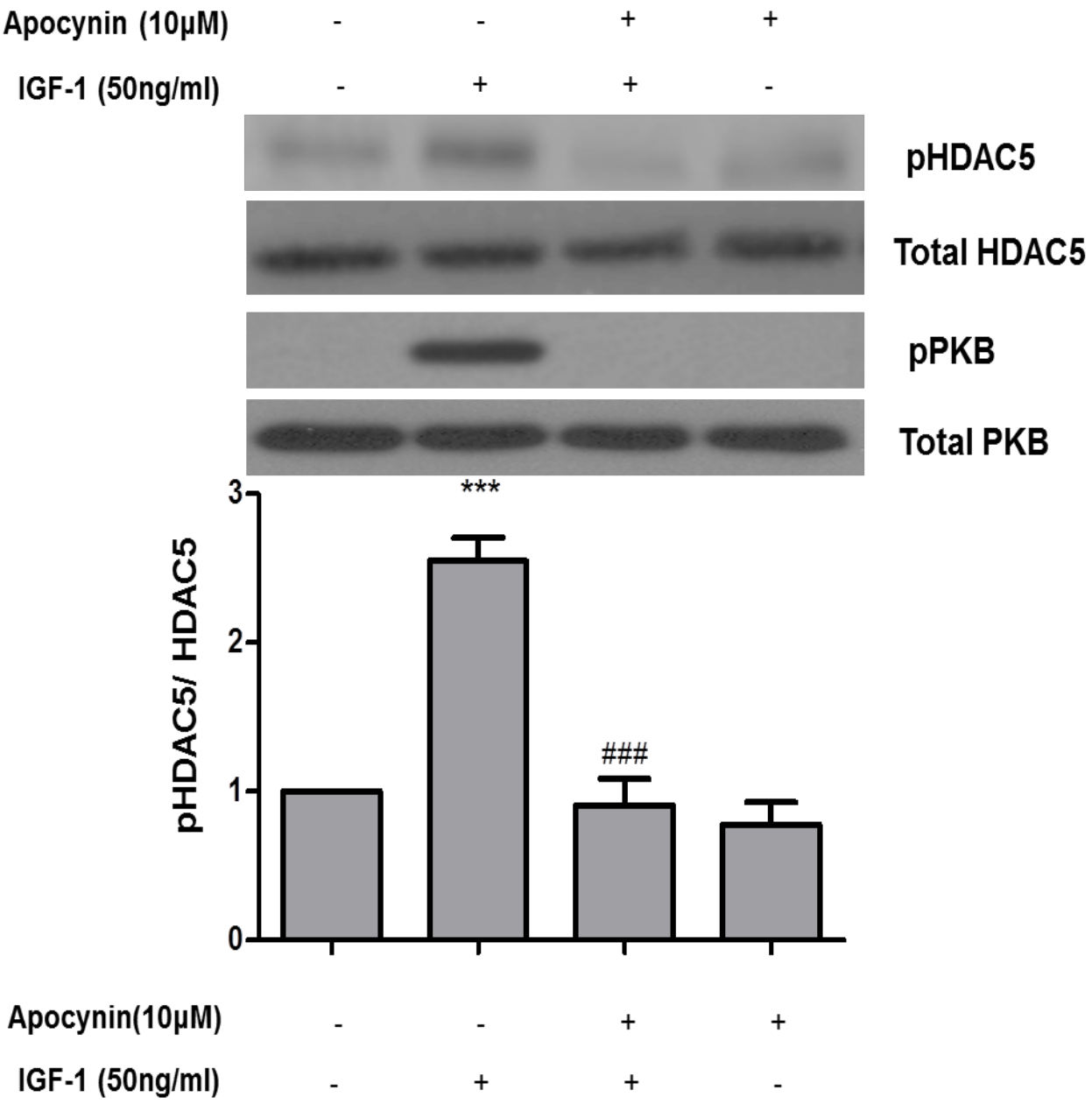

Apocynin (10μM) - - + +
IGF-1 (50ng/ml) - + + -
pHDAC5
Total HDAC5
pPKB
Total PKB
pHDAC5/ HDAC5
***
###
0
1
2
3
Apocynin(10μM) - - + +
IGF-1 (50ng/ml) - + + -

Figure 7

C)

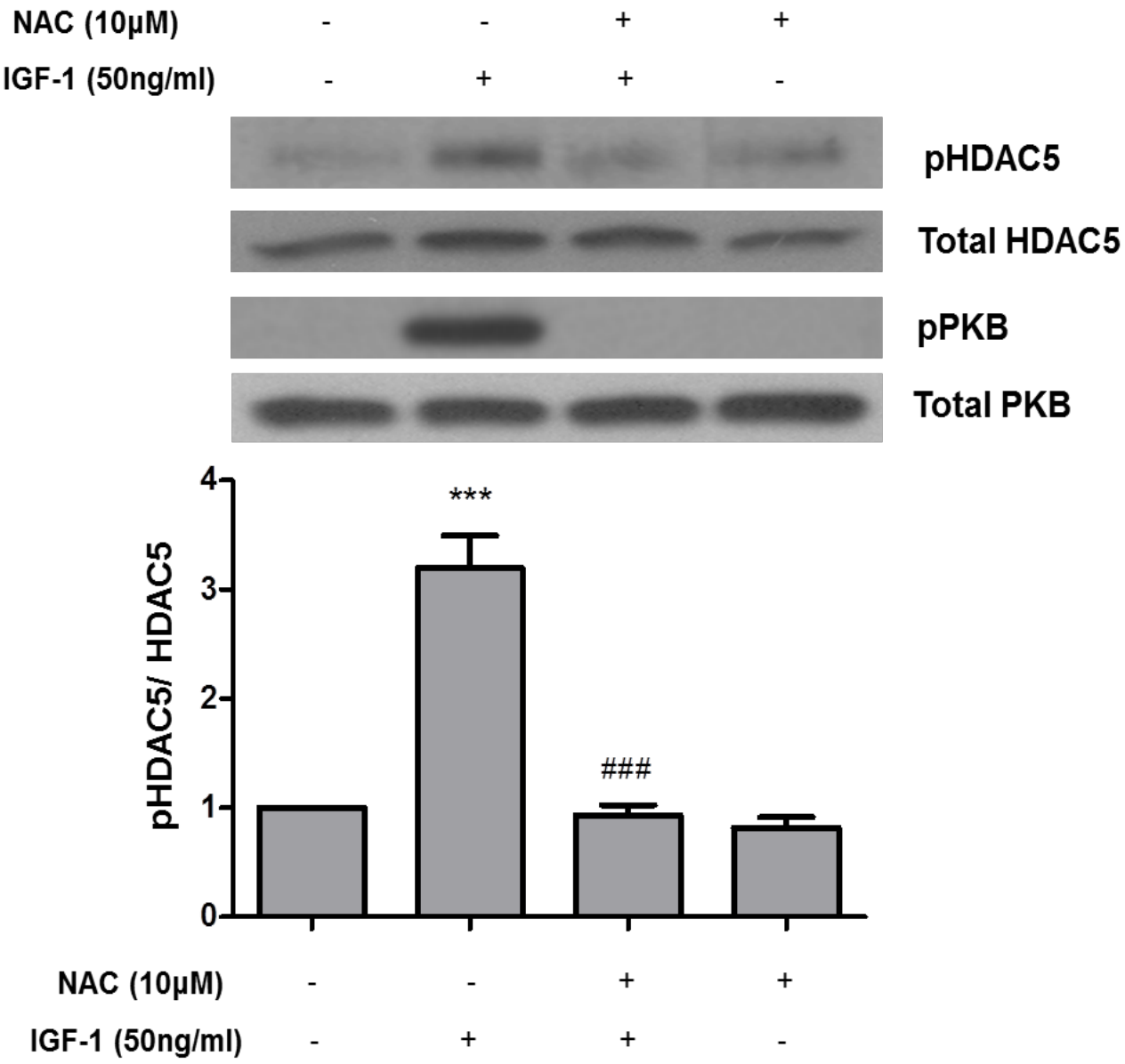

NAC (10μM) - - + +
IGF-1 (50ng/ml) - + + -
pHDAC5
Total HDAC5
pPKB
Total PKB
pHDAC5/ HDAC5
0
1
2
3
4
***
###
NAC (10μM) - - + +
IGF-1 (50ng/ml) - + + -

Figure 8

A)

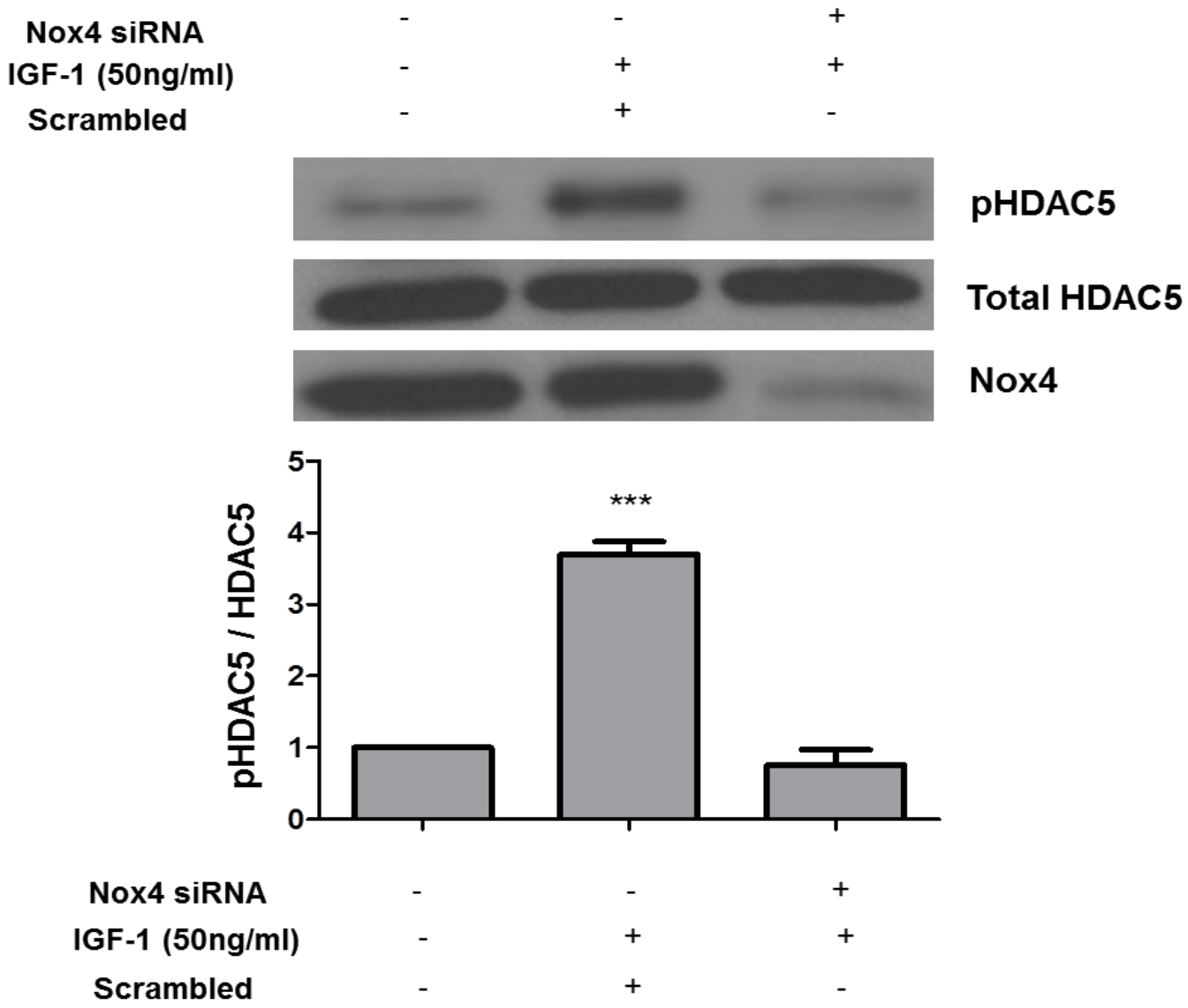

Nox4 siRNA - - +
IGF-1 (50ng/ml) - + +
Scrambled - + -
pHDAC5
Total HDAC5
Nox4
pHDAC5 / HDAC5
0
1
2
3
4
5
***
Nox4 siRNA - - +
IGF-1 (50ng/ml) - + +
Scrambled - + -

Figure 8

B)

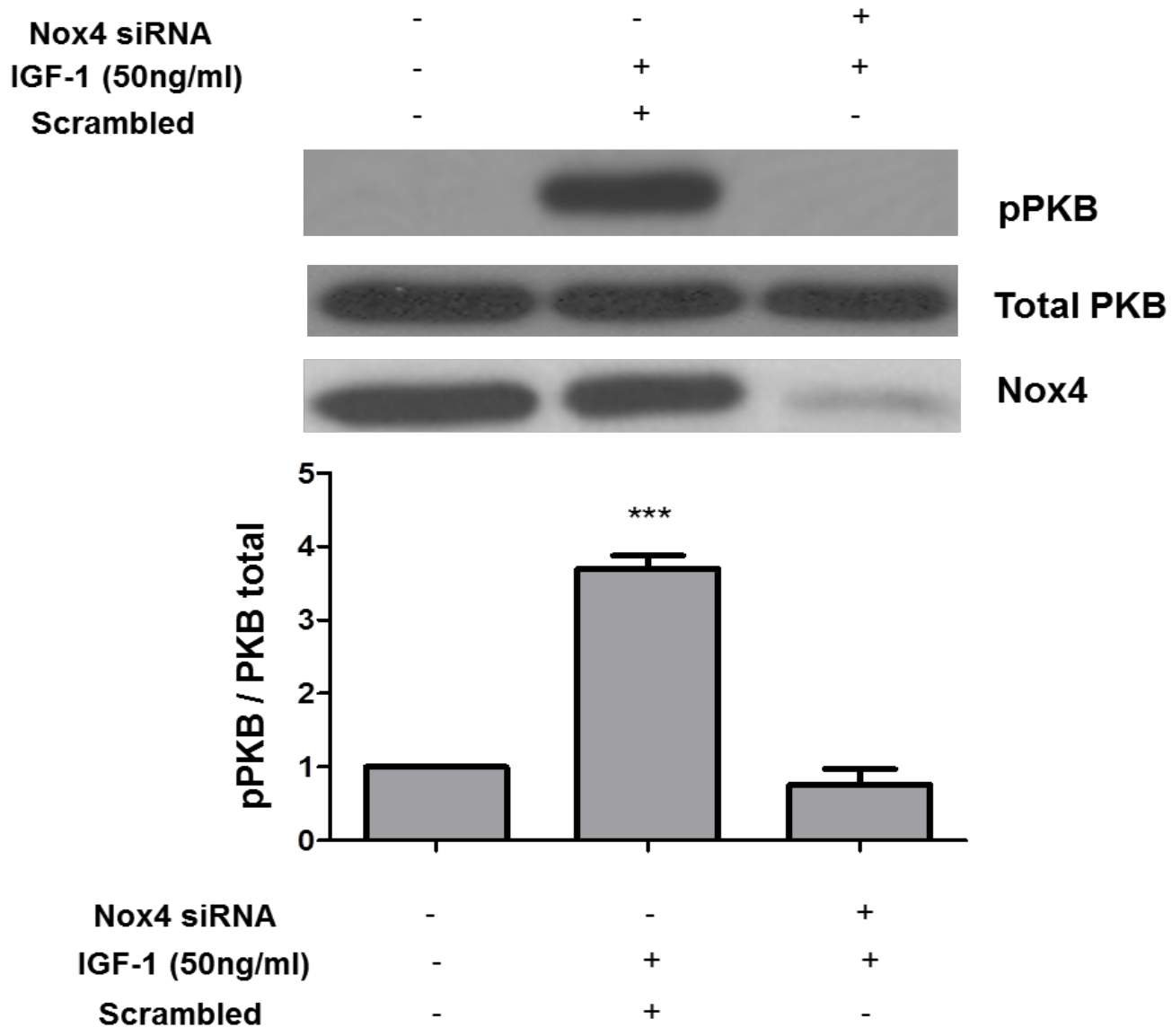

Nox4 siRNA - - +
IGF-1 (50ng/ml) - + +
Scrambled - + -
pPKB
Total PKB
Nox4
pPKB / PKB total
0
1
2
3
4
5
***
Nox4 siRNA - - +
IGF-1 (50ng/ml) - + +
Scrambled - + -

Figure 9

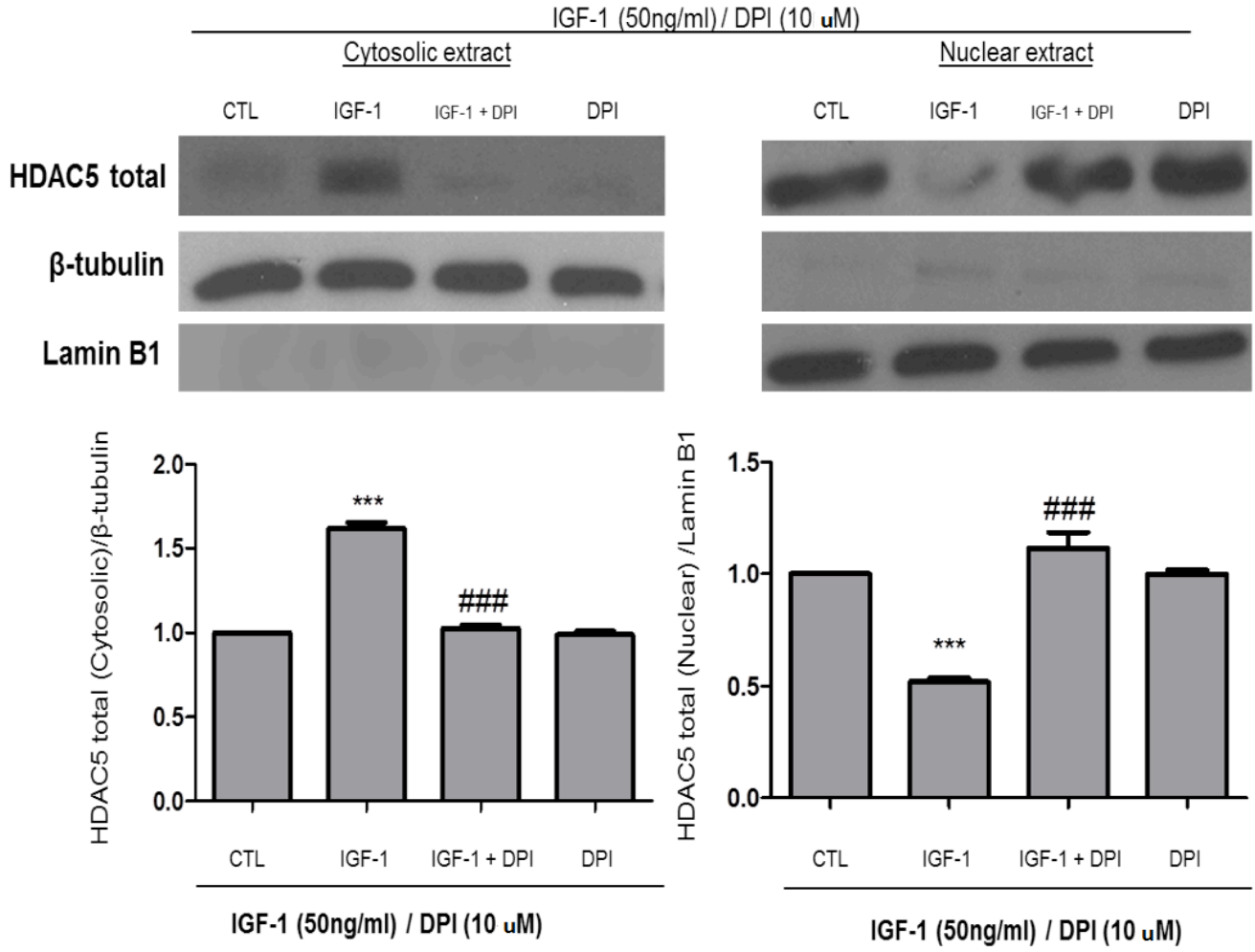
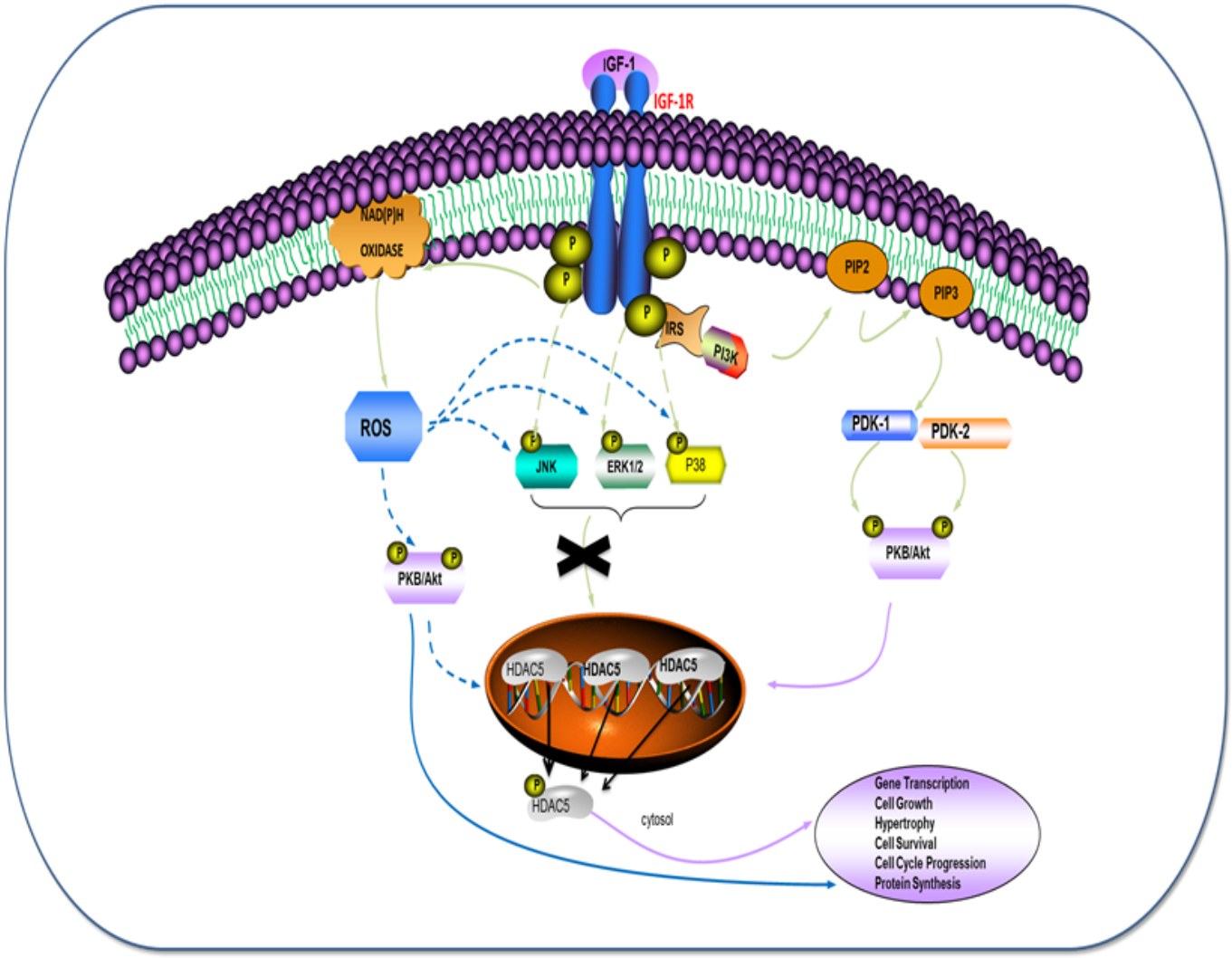

IGF-1 (50ng/ml) / DPI (10 uM)
Cytosolic extract
Nuclear extract
CTL
IGF-1
IGF-1 + DPI
DPI
HDAC5 total
β-tubulin
Lamin B1
HDAC5 total (Cytosolic)/β-tubulin
2.0
1.5
1.0
0.5
0.0
***
###
HDAC5 total (Nuclear) /Lamin B1
IGF-1 (50ng/ml) / DPI (10 uM)

Figure 10


IGF-1
IGF-1R
NAD(P)H
OXIDASE
P
P
P
P
IRS
PI3K
PIP2
PIP3
ROS
JNK
ERK1/2
P38
PDK-1
PDK-2
PKB/Akt
PKB/Akt
HDAC5
HDAC5
HDAC5
HDAC5
cytosol
Gene Transcription
Cell Growth
Hypertrophy
Cell Survival
Cell Cycle Progression
Protein Synthesis

# CHAPTER 3

# GENERAL DISCUSSION

The main feature of CVD is an increased vascular resistance due to blood vessel remodeling (2). Blood vessels are lined by VSMCs, which are highly specialized cells that regulate vascular tone and blood pressure (3). Abnormal migration, growth and proliferation of VSMCs are believed to be key events that contribute to structural remodeling of blood vessels and have been attributed to the pathogenesis of vascular diseases such as hypertension and atherosclerosis (3). A potential role of vasoactive peptides and growth factors, such as IGF-1, in inducing proliferation, hypertrophy, migration and differentiation of VSMCs has been demonstrated (5, 6, 7). IGF-1 binds to its receptor IGF-1R, a transmembrane tyrosine kinase that is greatly expressed in VSMCs, enabling IGF-1 to carry out its mitogenic effects (15). Once IGF-1R is activated, two main signaling pathways are triggered giving rise to IGF-1's growth promoting properties. The first signaling cascade is the mitogen activated protein kinase (MAPK) pathway, which includes extracellular signal-regulated kinase 1/2 (ERK1/2), p38 MAPK and c-jun-NH2 terminal kinase (JNK). ERK1/2, p38 MAPK and JNK have all been shown to participate in the cellular events that lead to aberrant VSMC growth and migration and hyperactivation of these signaling molecules is observed in various models of hypertension and atherosclerosis (15, 59, 60). The second well-known pathway triggered after IGF-1R activation in VSMCs is the phosphatidyl-inositol 3-kinase/protein kinase B (PI3-K/PKB) pathway (15,66). Additionally, IGF-1R activation can also lead to upregulated ROS generation via NAD(P)H Oxidase (Nox) activation, triggering the MAPK and PKB pathways and thus representing another important player in cardiovascular disease progression (15). The major isoforms of Nox expressed in VSMCs are Nox1 and Nox4 (183); however, Nox4-derived ROS has been shown to be required for IGF-1’s pathophysiological actions in the vasculature (184).

In terms of HDAC5's role in vascular disease, HDAC5 has been shown to be an important regulator of gene activity and has been linked to the pathogenesis of several cardiometabolic diseases including hypertension, atherosclerosis and diabetes (157). Studies have reported a role of angiotensin II (AngII) as well as platelet derived growth factor (PDGF) in mediating HDAC5 phosphorylation and activation in VSMCs (110, 111). In terms of the signaling pathway implicated in this process, it has been illustrated that CaMKII is capable of mediating AngII- and PDGF-dependent increases in HDAC5 phosphorylation (154, 156). Additionally, protein kinase D1 (PKD1), which is activated in a protein kinase C (PKC)-dependent manner following AngII stimulation, has also been shown to mediate HDAC5 phosphorylation (157). IGF-1, like AngII and PDGF, has been shown to promote VSMC migration and proliferation (15), thus contributing to the development of vascular pathologies. However, the effect of IGF-1 on HDAC5 phosphorylation and activation in VSMCs remains unexplored and the pathways that link IGF-1 to HDAC5 phosphorylation still need to be unraveled.

In these studies, we examined the effect of IGF-1 on HDAC5 phosphorylation and nuclear export in VSMCs and determined the signaling pathways involved in this response. First, we demonstrated that IGF-1 stimulates HDAC5 phosphorylation in a time- and dose- dependent fashion. Then, we demonstrated that pharmacological blockade of the MAPK pathway with PD98059, UO126, SP600125 and SB203580 had no effect on HDAC5 phosphorylation, whereas inhibitors of the PI3K/ PKB pathways, wortmannin and SC-66, almost completely attenuated IGF-1-induced HDAC5 phosphorylation. In addition to immunoblotting data, these findings

were confirmed by immunofluorescence localization of phospho-HDAC5 and by siRNA-induced silencing of PKB. Our data also demonstrate that PKB-induced phosphorylation of HDAC5 contributed to its nuclear export, as shown through nuclear localization techniques. In addition, we demonstrated that pretreatment of A10 VSMCs with Diphenyleneiodonium (DPI) and apocynin, two NAD(P)H oxidase inhibitors, as well as the antioxidant N-Acetyl-Cysteine (NAC), resulted in an attenuation of IGF-1-induced phosphorylation of HDAC5 as well as PKB. We also demonstrated the involvement of ROS in IGF-1-mediated HDAC5 nuclear export through nuclear localization experiments using DPI. Finally, using siRNA-induced silencing of Nox4, we showed that IGF-1-induced HDAC5 phosphorylation is mediated through Nox4-derived ROS. To the best of our knowledge, the work presented here is the first to report an involvement of Nox4-derived ROS generation and PKB signaling in IGF-1-induced HDAC5 phosphorylation and nuclear export in VSMCs.

Taken together, our results demonstrate the involvement of the PI3-K/PKB pathway as well as Nox4-derived ROS generation as upstream regulators of HDAC5 phosphorylation in response to IGF-1. Since HDAC phosphorylation has been associated with enhanced migration and proliferation of VSMCs, it may be suggested that PKB-induced phosphorylation of HDAC5 in VSMCs could constitute a potential mechanism to trigger a signaling program leading to vascular remodelling. In terms of future experiments, a role of IGF-1 in HDAC4 phosphorylation and activation has not been investigated. Therefore, in future studies, it would be interesting to examine the effect of IGF-1 on the phosphorylation and activation of HDAC4 in VSMCs and identify the signaling pathways involved in this process.

# CHAPTER 4

# CONCLUSION

The results presented here demonstrate for the first time that the PI3-K/PKB pathway as well as Nox4-derived ROS generation are required in mediating IGF-1-induced HDAC5 phosphorylation and nuclear export in A10 VSMCs. These studies also demonstrated that the MAPK pathway was not involved in mediating IGF-1-induced HDAC5 phosphorylation in A10 cells. These results were obtained using PD98059, U0126, SP600125 and SB203580, inhibitors of ERK1/2, MEK1, JNK and p38 MAPK, respectively, as well as wortmannin and SC-66, inhibitors of PI3-K and PKB, respectively, and using apocynin and DPI, inhibitors of NAD(P)H oxidase, and the antioxidant NAC.

Studies have implicated IGF-1 in vascular remodeling through its promotion of VSMC proliferation, migration and hypertrophy. However, the precise mechanism by which IGF-1 contributes to its deleterious responses in the vasculature remains to be determined. Our studies demonstrated that the PI3-K/PKB pathway and ROS generation act as upstream regulators of HDAC5 phosphorylation in response to IGF-1, contributing to exaggerated induction of genes involved in vascular remodelling.

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