ADIPORON, A SMALL-MOLECULE ADIPONECTIN RECEPTOR AGONIST, INHIBITS VASCULAR SMOOTH MUSCLE CELL PROLIFERATION AND CONTRACTILE RESPONSE: MOLECULAR MECHANISMS AND THERAPEUTIC IMPLICATIONS

by

ARWA MOHAMMEDYOUSEF FAIRAQ

(Under the Direction of Lakshman Segar)

ABSTRACT

Obesity is associated with insulin resistance and an increase in the risk of cardiovascular disease, including atherosclerosis, restenosis after angioplasty, and hypertension. Adiponectin, one of the key adipokines released from adipose tissue, functions as an insulin sensitizer with anti-inflammatory and vasoprotective properties. Hypoadiponectinemia seen in obesity and type 2 diabetes is closely correlated with the development of vascular disease. Administration of adiponectin as a rational therapy is not practically feasible in a clinical setting due to its short half-life and high molecular mass. Alternative strategies may therefore include upregulation and/or activation of adiponectin receptors (AdipoR). Recently, Okada-Iwabu *et al.* reported the first orally active small-molecule AdipoR agonist, AdipoRon. It binds to and activates AdipoR1 and AdipoR2 subtypes leading to the activation of AMP-activated protein kinase (AMPK) and PPAR-α signaling pathways in liver and skeletal muscle. In high fat diet-fed mice, AdipoRon ameliorates insulin resistance, lowers plasma glucose level, increases fatty acid oxidation, and reduces oxidative stress, suggesting that AdipoRon is a viable treatment option for vascular disease. The present study is aimed at examining the likely beneficial effects of AdipoRon in the

vessel wall. Oral administration of AdipoRon (50 mg/Kg) in C57BL/6J mice significantly diminished arterial injury-induced neointima formation *in vivo*. Under *in vitro* conditions, AdipoRon treatment led to a significant inhibition of platelet-derived growth factor (PDGF)-induced vascular smooth muscle cell (VSMC) proliferation. From a mechanistic standpoint, AdipoRon enhanced AMPK activation and diminished basal and PDGF-induced phosphorylation of mTOR and its downstream targets, including p70S6K/S6 and 4E-BP1 in VSMCs. However, siRNA-mediated AMPK downregulation resulted in persistent inhibition of p70S6K/S6 phosphorylation, suggesting AMPK-independent effects for AdipoRon inhibition of mTOR signaling. Furthermore, in *ex vivo* studies using endothelium-denuded rat aorta, AdipoRon treatment led to an increase in AMPK phosphorylation with an accompanying decrease in agonist-induced smooth muscle contractility. Pretreatment with compound C (an AMPK inhibitor) did not affect AdipoRon inhibition of smooth muscle contractility, suggesting that AMPK activation does not contribute to AdipoRon-mediated vasorelaxant response. Together, the present findings suggest that the orally-administered AdipoRon has the potential to limit restenosis after angioplasty and promote vasodilation independent of AMPK activation in insulin-resistant state.

INDEX WORDS: AdipoRon; adiponectin, arterial injury, vascular smooth muscle cells, AMPK , mTOR, PDGF, contractility; vasorelaxation

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by

ARWA MOHAMMEDYOUSEF FAIRAQ

Pharm.D, King Abdul Aziz University, Jeddah, Saudi Arabia, 2007

Pharm.D, Massachusetts College of Pharmacy and Health Science, Boston, USA, 2012

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by

ARWA MOHAMMEDYOUSEF FAIRAQ

Major Professor:	Lakshman Segar
Committee:	Adviye Ergul
	Jiliang Zhou
	Somanath Shenoy

Electronic Version Approved:

Suzanne Barbour
Dean of the Graduate School
The University of Georgia
May 2017

DEDICATION

I dedicate this thesis to my family who gave me all support to achieve this dream.

My father and mother

(MohammedYousef and Ltifah)

whose love, encouragement and prayers make me successful in my life

My beautiful sisters (Nermeen, Areej, Abrar and Ethar)

My bother-in-law (Yaser)

without their inspiration, I wouldn't be able to circumvent all difficulties throughout my life

My nephews and nieces

who gave me the joy in the world

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

Restenosis after angioplasty

An estimated 500,000 patients in the U.S. undergo coronary angioplasty procedures every year [1]. Around 17.3% of these patients will develop coronary in-stent restenosis despite the use of drug-eluting stents [2]. Thus, restenosis remains a major complication of coronary angioplasty. In-stent restenosis is characterized by neointima formation, which is associated with phenotypic transition of vascular smooth muscle cells (VSMCs) from contractile-to-proliferative state [3]. Drug-eluting stents (e.g., sirolimus/rapamycin) that are in clinical use target VSMC proliferation and migration to limit restenosis [4]. Nevertheless, the use of sirolimus is associated with adverse effects such as glucose intolerance and induction of diabetes, as evidenced in experimental animal models [5, 6]. Hence, there is a need to develop newer drugs to decrease the risk of restenosis after angioplasty in vulnerable subjects.

Role of Vascular Smooth Muscle Cells in Atherosclerosis and Restenosis after Angioplasty

The arterial wall consists of three layers: tunica intima, media, and adventitia. The intimal layer consists of continuous endothelial monolayer, while the underlying media contains mainly VSMCs. The outer adventitia contains fibroblasts in a loose connective tissue, as well as small blood vessels and fat [7]. VSMCs are distinct from skeletal muscle in that they are not terminally differentiated and characterized by phenotypic plasticity [8]. VSMCs have the ability to change

their phenotype between contractile and synthetic states in response to environmental factors. In healthy blood vessels, VSMCs exhibit a contractile phenotype where they express contractile proteins such as smooth muscle (SM) α□actin and calponin [9] and characterized by low rates of proliferation, migration, and extracellular matrix (ECM) secretion [9].

In restenosis after angioplasty, vessel injury leads to neointima formation as a result of endothelial denudation following balloon inflation or stent placement [7, 10]. Loss of endothelial layer increases the platelet-derived growth factor (PDGF) release from platelets [11], which acts as a chemoattractant factor. Thus smooth muscle cells migrate into the neointima and proliferate and results in neointima formation [7].

Adipose tissue and adipokines: Adipo-vascular axis

Obesity is associated with an increased risk of insulin resistance and cardiovascular diseases, including hypertension, atherosclerosis, and restenosis after angioplasty [12, 13]. Adipose tissue is considered an endocrine gland that secretes various bioactive molecules called adipokines such as tumor necrosis factor-α (TNFα), monocyte chemoattractant protein-1 (MCP-1), interleukin-6 (IL-6) and leptin [14-17]. These adipokines participate in inflammation process, induction of insulin resistance and vascular dysfunction. Unlike the aforementioned adipokines, adipose tissue-derived adiponectin has antiinflammatory, insulin-sensitizing and vascular protective properties [18-20]. A decrease in adiponectin production/release is associated with the development of vascular disease. Normal circulating level of adiponectin, comprising approximately 0.01–0.05% of plasma protein, ranges from 5 to 30 μg/ml [21, 22]. Plasma adiponectin concentration is significantly low in obesity, type 2 diabetes, cardiovascular disease, hypertension, and metabolic syndrome [23-26]. Thus, adiponectin links obesity to an increased

risk of vascular disease. In both men and women with low levels of adiponectin, there is an increase in the risk of type 2 diabetes as evidenced in a 6-year follow-up study [27]. Further studies reported an association between high plasma adiponectin and a decrease in the risk of myocardial infarction, hypertension and coronary artery disease [26, 28, 29]. To date, the exact role of adiponectin in cardiovascular and metabolic disorders is still not characterized.

Circulating forms of Adiponectin

Adiponectin was first identified in 1995 by three different groups [21, 22, 30]. Adiponectin is also known as Acrp30 [21], AdipoQ [30], apM1 [22], or GBP28 [31]. Structurally, it has two distinct domains: C-terminal collagen-like fibrous domain and complement C1q-like globular domain. It undergoes post-translational modification to form three major oligomeric circulatory forms: a low-molecular weight (LMW) trimer, amedium-molecular weight (MMW) hexamer, and a high-molecular weight (HMW) 12–18 multimers or can circulate as small fragment known as globular adiponectin [32, 33]. Little is known about Adiponectin secretion or oligomer formation. Formation of disulfide bond between cysteine residues, hydroxylation and glycosylation of lysine residues contribute to posttranslational modifications in the assembly and secretion of HMW adiponectin [32]. A recent study reported an association between high HMW adiponectin levels with lower risk of CVD in middle-aged adults with high blood glucose [34].

Vascular disease and Adiponectin

Adiponectin-KO mice show a significant increase in neointimal thickening in the femoral artery injury model [35] and cuff-injury model [36] through the suppressive effect of adiponectin on vascular smooth muscle cells proliferation and migration. Furthermore,

adenovirus-mediated delivery of full-length adiponectin significantly reduces neointimal thickening in the injury femoral artery in adiponectin-KO mice [35] and ameliorates atherosclerotic lesion in apoE-KO mice [37]. Clinically, hypoadiponectinemia is strongly correlated with a higher incidence of vascular disease. Low plasma concentrations of adiponectin is significantly associated with lesion formation in patients with coronary artery disease (CAD) [38, 39], and predicts in-stent restenosis in patients undergoing coronary stenting [40, 41]. Among all adiponectin isoforms, high molecular weight adiponectin (HMW) exhibits the strongest anti-atherogenic effects [39, 42]. Low level of circulating HMW adiponectin is associated with a significant increase in coronary plaque vulnerability [43], increase coronary atherosclerosis severity [44] and cardiovascular complications [45]. These findings propose a protective action of against different vascular disorders, such as endothelial dysfunction, atherosclerosis and restenosis after angioplasty. Underlying mechanisms include the following:

Role of adiponectin in endothelial cells

Adiponectin has been shown to improve endothelial function in the vessel wall. Ex-vivo studies demonstrate that globular adiponectin promote dose-dependent vasorelaxation in the rat aorta [46]. In vitro studies show that adiponectin inhibits endothelial apoptosis and proliferation [47, 48]. In bovine aortic endothelial cells (BAEC) and human umbilical vein endothelial cells (HUVEC), recombinant globular adiponectin increases NO production via two main mechanisms. First, it activates endothelial nitricoxide synthase (eNOS) [47, 49] through phosphorylation of eNOS at Ser 1179 by an AMPK dependent mechanism [50]. It also increases eNOS binding to heat shock protein 90 (HSP90) to increase its activity [46]. Second, adiponectin also enhances eNOS expression [49]. Furthermore, unlike rapamycin, Adiponectin maintains Akt

signaling in endothelial cells. While adiponectin promotes VSMC differentiation, it prevents endothelial cell injury. Thus, targeting adiponectin pathway may offer an advantage over rapamycin toward the development of new drug-eluting stent therapy [51].

Role of adiponectin in vascular smooth muscle cells (VSMCs)

a. Adiponectin and PDGF

At physiological concentrations, adiponectin significantly reduces VSMC proliferation and migration induced by platelet-derived growth factor-BB (PDGF-BB). At the molecular level, adiponectin binds to PDGF-BB with a binding capacity of 2 ng PDGF-BB/μg adiponectin [19, 46] and significantly inhibits the association of PDGF-BB with PDGF receptors. As a result, Adiponectin suppresses PDGF receptor-β autophosphorylation and decreases p42/44 extracellular signal–related kinase (ERK) phosphorylation induced by PDGF-BB [35].

b. Adiponectin and adiponectin receptors

AdipoR1 and AdipoR2 serve as the main receptors for adiponectin. Each type of adiponectin receptor has a specific distribution with different affinities. Generally, AdipoR1 has high affinity to globular adiponectin and is expressed mainly in skeletal muscle. AdipoR2 has intermediate affinity for globular and HMW adiponectin and is most abundantly expressed in the liver. AdipoR1 and AdipoR2 have seven transmembrane domains but are functionally distinct from G-protein-coupled receptors with N-terminus internally and C-terminus externally. C-terminal domain mainly interacts with adiponectin, while N-terminal interacts with APPL (adaptor protein containing pleckstrin homology domain, phosphotyrosine-binding domain, and leucine zipper

motif) to activate downstream signaling pathways including AMP-activated protein kinase (AMPK) or PPARα activation *via* AdipoR1 and AdipoR2, respectively, in skeletal muscle and liver to promote glucose uptake and fatty acid oxidation [52]. AMPK acts as an energy sensor, and is a heterotrimeric complex with serine/threonine kinase activity [53]. It consists of three subunits including α-catalytic subunit and β and γ-regulatory subunits [54]. AMPK inhibits VSMC proliferation through inhibition of ERK [55], upregulation of cell cycle inhibitors such as p21Cip1 and p27 Kip1 [56] or inhibition of mTOR/p70S6K signaling [57]. In VSMCs, HMW adiponectin activates AMPK via Thr172 phosphorylation through adipoR1[58]. The increase in AMPK activity results in the phosphorylation of tuberous sclerosis complex 2 (TSC2) protein, leading to an inhibition of mammalian target of rapamycin complex 1 (mTORC1) activity and protein synthesis. Studies by Martin and co-workers have shown that adiponectin-mediated inhibition of mTORC1 promotes VSMC differentiation [51].

T-cadherin, a glycosyl-phosphatidylinositol (GPI)-anchored cadherin cell surface glycoprotein, may also act as a receptor for adiponectin specifically for hexameric and HMW forms of adiponectin but not for trimeric or globular adiponectinhug T-cadherin is expressed in the heart, vascular smooth muscle and endothelium [59]. In vivo studies have shown that adiponectin and T-cadherin proteins are co-localized in aorta, heart, and skeletal muscle [60]. In Adipo KO mice, Tcad protein was highly expressed in cardiac and vascular tissues. However, no significant difference is reported in Tcad mRNA levels suggesting that Tcad is regulated by adiponectin at the posttranslational level. In Tcad KO mice, adiponectin concentration is significantly high without any changes in adiponectin synthesis [59, 60]. In another study, in relation to the neointima and atherosclerotic plaque lesions, T-cad/ApoE-DKO mice increased atherosclerotic plaque formation compared with ApoE/KO mice [61]. T-cadherin presents in

synthetic SMCs, and thus leads to adiponectin accumulation to mediate its vascular protective role [61]. To date, the role of T-cadherin in the vasoprotective effect of Adiponectin remains elusive.

c. Role of adiponectin in atherosclerosis

Atherosclerosis is characterized by monocyte adhesion to endothelial cells, oxidized LDL uptake by macrophages, and smooth muscle cell migration and proliferation. Physiological adiponectin inhibits the expression of adhesion molecules, including intracellular adhesion molecule-1, vascular cellular adhesion molecule-1, and E-selectin [62]. Furthermore, adiponectin inhibits the TNFα-induced nuclear factor-κB activation, which leads to inhibition of monocyte adhesion to endothelial cells [63]. Second, adiponectin inhibits the expression of the scavenger receptor class A-1 (SR-A) of macrophages, causing a decrease in oxidized LDL uptake and inhibition of foam cell formation [64]. Third, adiponectin inhibits SMC proliferation via binding to platelet-derived growth factor-BB receptor [19]. Together adiponectin exhibits antiatherogenic effects.

Limitations of adiponectin therapy - AdipoRon as a possible alternative

Regardless of the beneficial effects of adiponectin, clinical administration of adiponectin is still not practically feasible. First, it cannot be administered orally since it is a 30 kDa protein. Although the production of recombinant human adiponectin protein in bacteria was successful [47, 50], it still requires several posttranslational modification steps (to form LMW, MMW, HMW), which occur only if produced in mammalian cells. Thus, alternative strategies to achieve the benefit from Adiponectin include an increase in circulating adiponectin level indirectly via

pharmacological agents such as thiazolidinediones (TZD) or ACEI or via weight loss and physical exercise or mediterranean-type diet [65, 66]. Another strategy is to up-regulate or activate adiponectin receptors. In 2013, Okada-Iwabu et al. discovered the first orally active small molecule AdipoR agonist, AdipoRon [67], it binds to and activates AdipoR1 and AdipoR2 leading to activation of AMPK and PPAR-α pathways in liver and skeletal muscle. Similar to adiponectin, AdipoRon ameliorates insulin resistance in mice fed a high-fat diet, lower plasma glucose level, increases fatty acid oxidation, reduces oxidative stress and increases life expectancy, suggesting AdipoRon as a possible treatment for type 2 diabetes in the future [34]. Thus, the use of AdipoRon provides a promising therapeutic approach to target adiponectin pathway in vascular disease.

To date, there are few reports on AdipoRon action in cardiac/vascular tissues. Zhang et al. have shown that oral administration of AdipoRon (50 mg/kg, 10 min pior MI) improves cardiac function by ameliorating post-ischemic cardiac injury in wild-type mice [68]. Furthermore, AdipoRon prevents myocardial ischemia/reperfusion (MI/R)-induced cell apoptosis in Adiponectin knockout mice (APNKO) or cardiomyocyte-specific AMPK dominant-negative mice (AMPK-DN). Thus, adipoRon attenuates postischemic myocardial apoptosis via AMPK-dependent and AMPK-independent mechanisms [68]. In a different study, Hong et al have shown a direct vasorelaxant effect of adipoRon in smooth muscle as a function of concentration (1-50 μM). The vasorelaxant effect occurs independent of endothelium, AMPK activation, and cytosolic free calcium level [Ca2+] [69]. As a result, AdipoRon exhibits vasodilation effect through mechanisms distinct from adiponectin. These few studies suggest AdipoRon as a possible novel therapeutic molecule for cardiovascular disease.

Figure legends

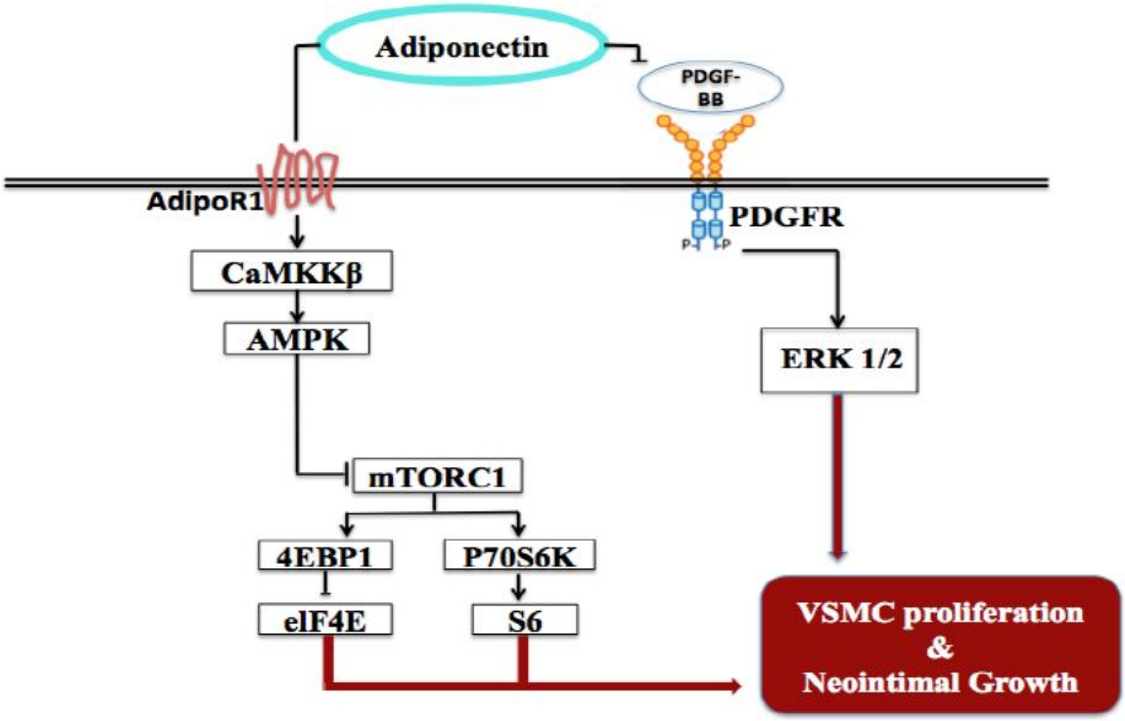
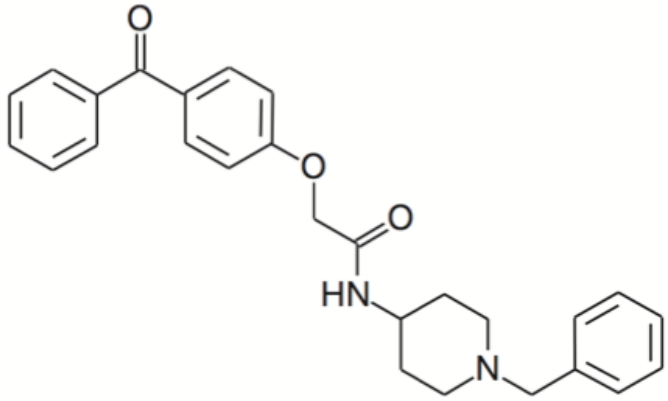


Fig. 1.1. Adiponectin suppresses VSMC proliferation *via* two main mechanisms that include activation and inhibition of AdipoR1 and PDGFR signaling pathways, respectively.



AdipoRon
Formula: $C_{27}H_{28}N_2O_3$
Synonyms: 2-(4-Benzoylphenoxy)-N-[1-(phenylmethyl)-4-piperidinyl]acetamide
Molecular weight: 428.5

Fig. 1.2. Chemical structure of AdipoRon [67]

Problem Statement and Specific Aims

Obesity and type 2 diabetes are associated with an increased risk of vascular complications including atherosclerosis and restenosis after angioplasty. In particular, a decrease in the circulating concentration of adipose tissue-derived adiponectin is closely correlated with insulin resistance and coronary artery disease. Previous studies demonstrate that adenovirus-mediated adiponectin delivery attenuates atherosclerosis in apoE-deficient mice and inhibits injury-induced neointima formation in adiponectindeficient mice [35, 37], suggesting a vasoprotective role for adiponectin. Nevertheless, the use of exogenous adiponectin as a rational therapy to reduce cardiovascular disease risk has been hampered in a clinical setting due to its large molecular size and short plasma half-life. A recently developed small molecule adiponectin receptor agonist (AdipoRon), when administered orally, is found to improve insulin resistance and glycemic control in a type 2 diabetic mouse model [67]. The beneficial effects of Adiporon have been attributed in part to activation of AMP-activated protein kinase (AMPK), which is characteristic of endogenous adiponectin. To date, the likely beneficial effects of AdipoRon have not yet been examined in the vessel wall.

Driven by my long-term goal to identify novel therapeutic strategies to limit restenosis after angioplasty, the objective of the research proposal is to determine the molecular mechanisms by which AdipoRon regulates vascular smooth muscle cell (VSMC) phenotype in vitro and in vivo.

My central hypothesis is that AdipoRon inhibits VSMC proliferation and contractile response through AMPK-dependent and AMPK-independent mechanisms. There are three specific aims:

Specific Aim I: To determine the extent to which orally-delivered AdipoRon regulates neointima formation in the mouse model of arterial injury. If Adiporon inhibits neointima

formation under normal metabolic milieu, it would imply that it has a direct inhibitory effect on VSMC proliferation in the injured vessel wall.

Specific Aim II: To determine the molecular mechanisms by which AdipoRon regulates VSMC proliferation. Serum-deprived human aortic VSMCs in culture are exposed to AdipoRon as a function of concentration and time to determine its likely regulatory effects on: i) VSMC proliferation. ii) PDGF receptor-mediated proliferative signaling events including mTOR/p70S6kinase; and iii) crosstalk between AMPK and mTOR signaling. However, AMPK activation may or may not contribute to inhibition of PDGF-induced key proliferative signaling.

Specific Aim III: To determine the intermediary role of AMPK in AdipoRon-mediated vasorelaxation. Contractility studies using endothelium-denuded rat aorta will allow us to examine the intermediate role of AMPK activation in AdipoRon-mediated smooth muscle relaxation.

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CHAPTER 2

ADIPORON, AN ADIPONECTIN RECEPTOR AGONIST, ATTENUATES PDGF-INDUCED VSMC PROLIFERATION THROUGH INHIBITION OF mTOR SIGNALING INDEPENDENT OF AMPK: IMPLICATIONS TOWARD SUPPRESSIONS OF NEOINTIMAL HYPERPLASIA

Arwa Fairaq, Noha M. Shawky, Islam Osman, Prahalathan Pichavaram, Lakshman Segar. 2017, Pharmacological Research, 289-302

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ABSTRACT

Hypoadiponectinemia is associated with an increased risk of coronary artery disease. Although adiponectin replenishment mitigates neointimal hyperplasia and atherosclerosis in mouse models, adiponectin therapy has been hampered in a clinical setting due its large molecular size. Recent studies demonstrate that AdipoRon (a small-molecule adiponectin receptor agonist) improves glycemic control in type 2 diabetic mice and attenuates postischemic cardiac injury in adiponectin-deficient mice, in part, through activation of AMP-activated protein kinase (AMPK). To date, it remains unknown as to whether AdipoRon regulates vascular smooth muscle cell (VSMC) proliferation, which plays a major role in neointima formation. In the present study, oral administration of AdipoRon (50 mg/Kg) in C57BL/6J mice significantly diminished arterial injury-induced neointima formation by ~57%. Under *in vitro* conditions, AdipoRon treatment led to significant inhibition of platelet-derived growth factor (PDGF)-induced VSMC proliferation, DNA synthesis, and cyclin D1 expression. While AdipoRon induced a rapid and sustained activation of AMPK, it also diminished basal and PDGF-induced phosphorylation of mTOR and its downstream targets, including p70S6K/S6 and 4E-BP1. However, siRNA-mediated AMPK downregulation showed persistent inhibition of p70S6K/S6 and 4E-BP1 phosphorylation, indicating AMPK-independent effects for AdipoRon inhibition of mTOR signaling. In addition, AdipoRon treatment resulted in a sustained and transient decrease in PDGF-induced phosphorylation of Akt and ERK, respectively. Furthermore, PDGF receptor-β tyrosine phosphorylation, which controls the phosphorylation state of Akt and ERK, was diminished upon AdipoRon treatment. Together, the present findings suggest that orally-administered AdipoRon has the potential to limit restenosis after angioplasty by targeting mTOR signaling independent of AMPK activation.

Keywords:

AdipoRon

AMPK

mTOR

PDGF

Vascular smooth muscle cells

Arterial injury

Abbreviations:

VSMCs, vascular smooth muscle cells; PDGF, platelet-derived growth factor; AMPK, AMP-activated protein kinase; ACC, acetyl CoA carboxylase; mTOR, mammalian target of rapamycin; p70S6K, p70 ribosomal protein S6 kinase; S6, S6 ribosomal protein; 4E-BP1, eukaryotic initiation factor 4E-binding protein 1; ERK, extracellular signal-regulated kinase; PI 3-kinase, phosphatidylinositol-4,5-bisphosphate *3-kinase;* GAPDH, glyceraldehyde 3-phosphate dehydrogenase; siRNA, small interfering RNA; EVG, elastic van gieson; H&E, haematoxylin and eosin.

1. Introduction

Hypoadiponectinemia is closely correlated with insulin resistance and coronary artery disease [1-4]. In adiponectin-deficient mice, a decrease in the circulating concentration of adiponectin leads to insulin resistance and ~2-fold increase in neointima formation after arterial injury [5]. Of importance, adenovirus-mediated adiponectin delivery inhibits exaggerated neointima formation after arterial injury in adiponectin-deficient mice [6]. In addition, it attenuates atherosclerosis in apoE-deficient mice [7], suggesting a vasoprotective role for adiponectin. Nevertheless, the use of exogenous adiponectin as a rational therapy to reduce cardiovascular disease risk has been hampered in a clinical setting due to its large molecular size and short plasma half-life [8]. Recent studies demonstrate that oral administration of AdipoRon, a small-molecule adiponectin receptor agonist, improves insulin resistance and glycemic control in type 2 diabetic mice [9]. Furthermore, AdipoRon has been shown to attenuate post-ischemic myocardial apoptosis in adiponectin-deficient mice [10]. The beneficial effects of AdipoRon in skeletal muscle cells and cardiac myocytes have been attributed, in part, to activation of AMP-activated protein kinase (AMPK) [9, 10]. To date, the likely regulatory effects of AdipoRon toward neointimal hyperplasia *in vivo* and vascular smooth muscle cell (VSMC) proliferation *in vitro* have not yet been examined.

The circulating adiponectin exists as globular adiponectin, full-length adiponectin, or oligomeric forms including low molecular weight (LMW), medium molecular weight (MMW), and high molecular weight (HMW) adiponectin [11]. Of importance, adiponectin exhibits its insulin-sensitizing action through adiponectin receptor-mediated AMPK activation in insulin responsive tissues such as skeletal muscle and liver [12]. Studies by Martin and co-workers

demonstrate that in VSMCs, adiponectin-mediated AMPK activation leads to inhibition of mammalian target of rapamycin (mTOR)/p70S6kinase (p70S6K) signaling thereby promoting a differentiation phenotype [13]. In addition, studies by several investigators, including our recent observations, reveal that AMPK-mediated inhibition of mTOR/p70S6K signaling contributes in part to attenuation of VSMC proliferation [14-16]. However, the role of AdipoRon on AMPK activation and the resultant effects on VSMC proliferative signaling remain unknown.

In addition to activating AMPK [13], adiponectin has been shown to suppress platelet-derived growth factor (PDGF) receptor signaling in VSMCs [6, 17, 18]. In this regard, HMW adiponectin diminishes PDGF-induced VSMC proliferation through direct interaction with PDGF-BB [17, 18]. In addition, adiponectin inhibits the autophosphorylation of PDGF receptor-β and the phosphorylation of its downstream effector, extracellular signal-regulated kinase (ERK) in VSMCs [17]. Nevertheless, the role of AdipoRon on PDGF-induced key proliferative signaling events, including mTOR, Akt, and ERK, remains to be examined in VSMCs.

In the present study, we therefore hypothesize that AdipoRon attenuates VSMC proliferation through activation of AMPK and/or inhibition of PDGF-induced mTOR signaling. The specific objectives are to determine the extent to which AdipoRon regulates neointimal hyperplasia *in vivo* and VSMC proliferation *in vitro*. Using C57BL/6J mice, this study has determined the effects of orally-administered AdipoRon on neointima formation after femoral artery injury. Using human aortic VSMCs, this study has also determined the effects of AdipoRon on: i) cell proliferation, DNA synthesis, and cyclin D1 expression; ii) phosphorylation state of AMPK and its downstream target, acetyl CoA carboxylase (ACC); iii) PDGF-induced phosphorylation of mTOR and its downstream targets, including p70S6K/S6 ribosomal protein and 4E-BP1; iv) PDGF-induced mTOR signaling after target-specific downregulation of AMPKα1 isoform; v)

PDGF-induced phosphorylation of ERK1/2 (a downstream target of MEK1) and Akt (a downstream target of PI 3-kinase); and vi) PDGF receptor-β tyrosine phosphorylation and its association with p85 (an adapter subunit of PI 3-kinase).

2. Material and methods

2.1. Chemicals

AdipoRon was purchased from Cayman Chemical Company (Ann Arbor, MI). Recombinant human PDGF-BB was purchased from R&D Systems (Minneapolis, MN). The primary antibodies for phospho-PDGFRβ^{Tyr751} (3161), PDGFRβ (3169), phospho-AMPKα^{Thr172} (2535), pan-AMPKα (2532), phospho-ACC^{Ser79} (11818), ACC (3676), phospho-C-Raf^{Ser338} (9427), C-Raf (12552), phospho-MEK1/2$^{Ser217/221}$ (9154), MEK1/2 (8727), phospho-44/42 MAPK (pERK1/2; 4370), 44/42 MAPK (ERK1/2; 4695), phospho-Akt^{Thr308} (2965), phospho-Akt^{Ser473} (9271), Akt (4691), phospho-mTOR^{Ser2448} (5536), mTOR (2972), phospho-p70S6K^{Thr389} (9234), p70S6K (2708), phospho-4E-BP1^{Ser65} (9451), 4E-BP1 (9644), phospho-S6 ribosomal protein$^{Ser235/236}$ (4857), S6 ribosomal protein (2217), cyclin D1 (2922), β-actin (8457). GAPDH (5174), anti-rabbit IgG HRP-linked secondary antibody (7074), signal stain antibody diluent (8112), and signal stain boost IHC detection reagent (8114) were purchased from Cell Signaling Technology (Danvers, MA). Anti-mouse IgG secondary antibody (1706516) was purchased from Bio-Rad (Hercules, CA). The primary antibody for p85 (05217) was purchased from Millipore (Billerica, MA). AMPKα1 silencer select siRNA and scrambled siRNA were purchased from Life Technologies (Carlsbad, CA). Human aortic smooth muscle cells, vascular cell basal medium and vascular smooth muscle cell growth kit were purchased from ATCC

(Manassas, Virginia). The primary antibody for smooth muscle α-actin was purchased from Abcam (ab5694; Cambridge, MA). The primary antibody for Ki-67 (RM-9106-S1) and Mayer's hematoxylin (TA-125-MH) were purchased from Thermo Scientific (Wilmington, DE). Goat anti-rabbit secondary antibody (A-11037) and prolong gold anti-fade mountant with DAPI (P36931) were purchased from Life Technologies (Grand Island, NY). ImmPACT DAB peroxidase HRP substrate (SK-4105) was purchased from Vector laboratories (Burlingame, CA). All surgical tools were purchased from Roboz Surgical Instrument (Gaithersburg, MD). All other chemicals were from Fisher Scientific (Fair Lawn, NJ) or Sigma Chemical (St. Louis, MO).

2.2. Animals

All animal experiments were performed in accordance with the Charlie Norwood Veterans Affairs Medical Center Institutional Animal Care and Use Committee guidelines and were approved by the committee. Male C57BL/6J mice (12 weeks of age, 24-25 g, Jackson Laboratories, Bar Harbor, ME) were maintained in a room at a controlled temperature of 23°C with a 12:12-hr dark-light cycle. The mice had free access to water and rodent chow diet (8904; Harlan Teklad, Madison, WI).

2.3. Experimental protocol for AdipoRon treatment in mice

At 13 weeks of age, mice were divided into two groups. AdipoRon was suspended in 0.5% carboxymethyl cellulose and administered orally to the first group of mice (n = 7) at a dose of 50 mg/Kg/day for 21 days. A control group (n = 5) was kept in parallel where an equivalent volume of the vehicle was administered daily.

2.4. Femoral artery injury in mice

One day after AdipoRon or vehicle administration, left femoral artery injury was performed in all mice, as described [19, 20]. In brief, the left femoral artery was exposed by dissection and the femoral nerve was carefully separated. A small branch between rectus femoris and vastus medialis muscles was isolated, ligated distally, and looped proximally. After topical application of a drop of 1% lidocaine, transverse arterioctomy was applied in the muscular branch. An incision was made to allow the insertion of a straight spring wire (0.38 mm in diameter, No. C-SF-15-15, Cook, Bloomington, IN) into the femoral artery. The wire was left for 1 min in place to denude and dilate the artery and then removed. Suturing was performed in the proximal portion of the muscular branch to restore the blood flow in the main femoral artery. Right femoral artery was used as sham control.

2.5. Tissue collection

On day 21 (after femoral artery injury), mice were anesthetized with 2% isoflurane and then perfused *via* the left ventricle with 0.9% NaCl solution followed by 4% paraformaldehyde. The femoral artery was excised carefully and fixed in 4% paraformaldehyde for 1-2 days before being processed for frozen sections, as described [20].

2.6. Morphometric analysis of femoral artery

5 μm-thick cross-sections of the injured femoral artery were stained with haematoxylin and eosin (H&E) and elastic van gieson (EVG). The images were taken by AxioCam high resolution camera (HRc) attached to an Observer Z1 microscope (Carl Zeiss Microimaging, Inc., Thornwood, NY) using 20x magnification power. Cross-sectional arterial microscopic images

were analyzed for intima-to-media ratios within the middle portion of each lesion using image analysis software (Axiovision, release 4.8.2 SP3). The intimal area from each section was determined by subtraction of the lumen area from the internal elastic lamina (IEL) area. The medial area was determined by subtraction of the IEL area from the external elastic lamina (EEL) area, as described [20].

2.7. Immunofluorescence analysis of femoral artery

The cross sections of the injured femoral artery were subjected to immunofluorescence analysis for smooth muscle α-actin (SM α-actin) to confirm that the neointimal layer is composed of vascular smooth muscle cells. In addition, immunofluorescence analysis was performed for Ki-67, a marker of cell proliferation. The femoral artery sections were fixed in 4% paraformaldehyde and blocked by incubation with 5% normal goat serum for 1 hr. Subsequently, the sections were exposed to the primary antibody specific for SM α-actin (1:200 dilution) for 1 hr at room temperature or Ki-67 (1:30 dilution) overnight at 4°C. After washing 3 times with PBS, the sections were incubated with the secondary antibody (goat anti-rabbit IgG conjugated to Alexa fluor 594 with red fluorescence). The cross-sections were then mounted using prolong antifade with DAPI and the images were captured using confocal microscope at 20x magnification power, as described [20].

2.8. Immunohistochemical analysis of femoral artery

The cross sections of the injured femoral artery were subjected to immunohistochemical analysis to examine the phosphorylation state of S6 ribosomal protein, a downstream target mTOR/p70S6K signaling. The femoral artery sections were fixed in 10% neutral buffer formalin

and blocked by incubation with 5% normal goat serum for 1 hr. This was followed by incubation with the primary antibody specific for pS6 (1:75 dilution in signal stain antibody diluent) for 1 h at room temperature. After washing 3 times, hydrogen peroxide was added to block endogenous peroxidases and left for 10 min at room temperature. The sections were washed again 3 times and then incubated with signal stain IHC detection reagent for 30 min at room temperature. After intermittent washes, the sections were exposed to diaminobenzidine (DAB) working solution for 8 min. This was followed by counterstaining with Mayer`s hematoxylin. The sections were dehydrated by sequential immersions in 95% ethanol (2 times, 10 seconds each), 100% ethanol (2 times, 10 seconds each), xylene (2 times, 10 seconds each). They were then mounted using mounting medium (SP15-500, Fisher Scientific) and stored at room temperature. Images were captured using AxioCam high resolution camera (HRc) attached to an Observer Z1 microscope (Carl Zeiss Microimaging, Inc., Thornwood, NY) at 20x magnification power.

The intensity of pS6 staining in the neointima was quantified using Fiji software (ImageJ version 2.0.0-rc-15, National Institutes of Health). Reciprocal intensity, which was found to be directly proportional to antigen concentration, was calculated by subtracting the mean intensity in the neointimal area from the maximum intensity in white, non-stained background (250), as described [21].

2.9. Cell culture and treatments

Human aortic VSMCs were maintained in vascular cell basal medium, vascular smooth muscle cell growth supplement (SMGS), and antibiotic-antimycotic solution at 37°C in a humidified atmosphere of 95% air and 5% CO2, as described [22]. After the attainment of

confluence (~7-10 days), VSMCs (passages 3-4) were trypsinized, and seeded onto 60 mm petri dishes. Subconfluent VSMCs were maintained in medium devoid of serum (SMGS) for 48 hr to achieve quiescence, and then subjected to treatments as described in the respective legends. DMSO (0.1%) was used as vehicle control for AdipoRon treatment.

2.10. Alamar blue assay

The effect of AdipoRon on VSMC proliferation was initially assessed using Alamar blue substrate, as described previously [23, 24]. Serum-deprived VSMCs were incubated with increasing concentrations of AdipoRon (5, 25, 50 and 100 μM) for 96 hr. The medium and treatments were replenished at the 48 hr time point. The cells were exposed to Alamar blue (Invitrogen) during the last 4 hr. Alamar blue assay was performed according to manufacturer's instructions by measuring fluorescence (excitation/emission: 560/590 nm) using a spectrophotometer.

2.11. Cell counts

The effects of AdipoRon on basal and PDGF-induced VSMC proliferation were determined by cell counts using automated counter (Invitrogen), as described [22]. Serum-deprived VSMCs were incubated with increasing concentrations of AdipoRon (5, 25, 50 and 100 μM) for 30 min, and then exposed to PDGF (30 ng/ml) for 96 hr. The medium and treatments were replenished at the 48 hr time point. After 96 hr, the cells were washed twice with PBS, trysinized, and then counted.

2.12. DNA synthesis

The effects of AdipoRon on basal and PDGF-induced DNA synthesis were determined using click iT® EdU microplate assay according to manufacturer's instructions (Life technologies), as described [15]. Serum-deprived VSMCs were incubated with Adiporon (50 μM) for 24 hr and then exposed to PDGF (30 ng/ml) for the next 24 hr. During the last 18 hr of treatments, VSMCs were incubated with 5-ethynyl-2'-deoxyuridine (EdU; a nucleoside analog). Subsequently, cells were exposed to the supplied fixative reagent followed by labeling of EdU with green-fluorescent Oregon Green® azide. Signal amplification was achieved by incubation with HRP-conjugated anti-Oregon Green® antibody followed by reaction with Amplex® UltraRed substrate that produces a bright red fluorescent product (excitation/emission: 568/585 nm).

2.13. Immunoblot analysis

VSMC lysates (20 μg protein per lane) were subjected to electrophoresis using precast 4–12% NuPage mini-gels (Life Technologies), as described [22]. The resolved proteins were transferred to PVDF membranes (EMD Millipore). Subsequently, the membranes were blocked in 5% nonfat milk and probed with the respective primary antibodies. The immunoreactivity was detected using HRP-conjugated goat anti-rabbit secondary antibody (7074; Cell Signaling) followed by enhanced chemiluminescence (Thermo Scientific, Wilmington, DE). β-actin or GAPDH was used as internal controls. The protein bands were quantified using ImageJ.

2.14. Immunoprecipitation studies

Serum-deprived VSMCs were incubated with AdipoRon (50 μM) for 48 hr followed by exposure to PDGF (30 ng/ml) for 2 min. VSMC lysates were obtained using 1x RIPA buffer with protease inhibitor cocktail (P8340, Sigma Aldrich, St. Louis, MO) and phosphatase inhibitor cocktail (524629, EMD Millipore, Billerica, MA) The lysates were vortexed and then centrifuged at 5000 x g for 10 min at 4°C. Supernatants (200 μg protein) were incubated with anti-p85 primary antibody (1:50 dilution) overnight at 4°C, with continuous mixing. The antigen-antibody complexes were subjected to immunoprecipitation by mixing with magnetic beads (50 μL per sample) for 30 min at room temperature. After discarding the supernatants, the beads were washed three times with PBS containing 0.05% tween 20 (PBS-T). Elution step was performed by mixing the beads with 2x Laemmli buffer containing dithiothreitol and bromophenol blue and heating at 90°C for 10 min. The eluted samples were then used for immunoblotting.

2.15. Nucleofection of VSMCs with AMPKα1 siRNA

Subconfluent VSMCs were transfected with 500 pmoles of target-specific Silencer Select Pre-Designed siRNA (Life Technologies, Carlsbad, CA) using Amaxa Nucleofector-II device U-025 program (Lonza, Germany). Scrambled siRNA- and target-specific AMPKα1 siRNA-transfected VSMCs were incubated in complete medium for 48 hr. Subsequently, VSMCs were deprived of serum for 24 hr and then subjected to treatments as described in the figure legend.

2.16. Statistical analysis

The results are expressed as the means ± SEM. Statistical significance was tested using unpaired student t-test for morphometric analyses, AMPK α1 expression, and Akt, ERK and PDGFR-β phosphorylation (where only PDGF (0 μM AdipoRon) and PDGF (25 or 50 μM AdipoRon)-treated conditions were compared since the protein bands under control conditions were at undetectable intensities). One-way ANOVA and repeated measures one-way ANOVA followed by Bonferroni multiple comparisons tests were used for testing significance in concentration- and time-dependent studies, respectively. Two-way ANOVA followed by Bonferroni multiple comparisons tests were used for all other results in the study. Values of $p < 0.05$ were considered statistically significant. For siRNA data, the effects of AdipoRon treatment (AdipoRon *versus* vehicle) and PDGF exposure (PDGF *versus* control) were compared using regular two-way ANOVA followed by Bonferroni multiple comparisons test for each siRNA (Scr. and AMPK α1) separately. Values of $p < 0.05$ were considered statistically significant. Statistical analyses were carried out using GraphPad Prism software (GraphPad Software Inc. V6.0f, San Diego, CA, USA).

3. Results

3.1. Oral administration of AdipoRon attenuates neointima formation after arterial injury in mice.

Recent studies by Okada-Iwabu et al. have shown that oral administration of AdipoRon (50 mg/Kg) in C57BL/6 mice results in a maximal plasma concentration of ~12 μM [9]. In the

present study, a similar treatment protocol was followed in C57BL/6J mice by administering AdipoRon orally (50 mg/Kg) for 21 days.

As shown in **Fig. 2.1A-B**, H&E- and EVG-stained sections revealed a marked decrease in neointima formation in the injured femoral artery from AdipoRon-treated mice. In addition, morphometric analyses showed significant decreases in intima/media ratio and intimal area by ~63.2% and ~57.5%, respectively, in AdipoRon-treated group (**Fig. 2.1C**). There were no significant differences in the medial layer and lumen area between control and AdipoRon-treated groups (data not shown).

Immunofluorescence analysis of injured femoral artery sections showed a marked decrease in smooth muscle cells in the neointimal layer in AdipoRon-treated group, as revealed by SM α-actin immunoreactivity (**Fig. 2.2A**). Furthermore, AdipoRon treatment resulted in a marked decrease in Ki-67 immunoreactivity, a marker of cell proliferation (**Fig. 2.2B**).

3.2. AdipoRon inhibits basal and PDGF-induced VSMC proliferation

Previous studies have shown that adiponectin inhibits VSMC proliferation induced by PDGF-BB, a potent mitogen [6, 17, 18]. To determine the likely regulatory effects of AdipoRon on VSMC proliferation, serum-deprived VSMCs were incubated with AdipoRon at the indicated concentrations (5-100 μM) and time intervals with and without PDGF-BB exposure. AdipoRon concentrations in the range of 1-50 μM have been used in recent studies with rodent skeletal muscle arteries and cells [9, 25]. In the present study with human aortic VSMCs, there were significant decreases in cell number with AdipoRon treatment alone at 25 to 100 μM concentrations, as revealed by Alamar blue assay and cell counting using automated counter (**Fig. 2.3A and B**). PDGF-induced VSMC proliferation was diminished by > 60% upon

AdipoRon treatment at 5 to 100 μM concentrations. Furthermore, PDGF-induced increases in DNA synthesis and cyclin D1 expression were suppressed by AdipoRon pretreatment at the indicated concentrations (**Fig. 2.3C and D**).

3.3. AdipoRon enhances AMPK and ACC phosphorylation with an accompanying decrease in S6 phosphorylation in VSMCs

To determine the effects of AdipoRon on AMPK activation, concentration and time-dependent studies were performed to quantify AdipoRon-mediated changes in the phosphorylation state of AMPK and its downstream target, ACC. To determine the relationship between AMPK activation and mTOR signaling, these studies quantified the changes in the phosphorylation state of S6 ribosomal protein (a downstream target of mTOR/p70S6K). As shown in **Fig. 2.4A** and **B**, VSMC exposure to increasing concentrations of AdipoRon for 48 hr resulted in robust increases in $AMPK^{Thr172}$ phosphorylation. In particular, AdipoRon treatment at 25, 50 and 100 μM concentrations led to ~4.4, ~5.1 and ~6.7 fold increases in AMPK phosphorylation, respectively. In addition, AdipoRon treatment at similar concentrations resulted in ~5, ~7.5 and ~7.4 fold increases in the phosphorylation of ACC, a downstream target of AMPK (**Fig. 2.4A** and **B**). Notably, AdipoRon-mediated increases in the phosphorylation of AMPK and ACC were accompanied by diminutions in the phosphorylation of S6 ribosomal protein, which were observed at 25 to 100 μM concentrations (**Fig. 2.4A** and **B**). Time course studies with AdipoRon revealed a rapid rise in the phosphorylation of AMPK and ACC as early as 6 min with sustenance in the phosphorylation state of AMPK and ACC for a prolonged time interval (**Fig. 2.4C** and **D**). In addition, AdipoRon abolished S6 phosphorylation at 20 min to 6 hr time points (**Fig. 2.4C** and **D**).

3.4. AdipoRon inhibits basal and/or PDGF-induced acute phosphorylation of mTOR, p70S6K, S6, and 4E-BP1 in VSMCs

To further determine the effects of AdipoRon on mTOR signaling, AdipoRon-treated VSMCs were incubated with or without PDGF to quantify the changes in the phosphorylation state of mTOR and its downstream targets, including p70S6K/S6 ribosomal protein and 4E-BP1. As shown in **Figure 2.5A** and **B**, AdipoRon at 25 μM concentration diminished basal phosphorylation of mTOR by ~67% and completely abolished PDGF-induced phosphorylation of mTOR. In addition, AdipoRon abolished basal and PDGF-induced increases in the phosphorylation of p70S6K, pS6, and 4E-BP1.

3.5. Target-specific downregulation of AMPK does not affect AdipoRon-mediated diminutions in basal and PDGF-induced mTOR signaling in VSMCs

To determine the cause and effect relationship between AdipoRon-mediated AMPK activation and mTOR inhibition, VSMCs were nucleofected with AMPKα1 siRNA. As shown in **Fig. 2.6A and B**, nucleofection with AMPKα1 led to the downregulation of AMPK protein by 79.2%. Under these conditions, AdipoRon-mediated inhibition of basal and PDGF-induced phosphorylation of p70S6K, pS6, and 4E-BP1 and expression of cyclin D1 remained essentially the same as shown in **Fig. 2.6C and D**. These findings suggest that AdipoRon attenuates mTOR signaling in VSMCs independent of its role in AMPK activation.

3.6. AdipoRon inhibits basal and PDGF-induced phosphorylation of Akt in VSMCs

Previous studies have shown that sustained inhibition of mTOR signaling results in the activation of feedback regulatory loop involving activation of Akt [26]. To determine whether

AdipoRon inhibition of mTOR signaling has such an effect on Akt phosphorylation, AdipoRon-treated (3 hr or 48 hr) VSMCs were stimulated with PDGF for 6 min. As shown in **Fig. 2.7A-B**, AdipoRon treatment (25 μM, 3 hr) resulted in a significant decrease in PDGF-induced Akt^{Ser473} and Akt^{Thr308} phosphorylation by 44% and 29%, respectively. In addition, long-term exposure to AdipoRon (50 μM, 48 hr) led to a significant decrease in PDGF-induced Akt^{Thr308} phosphorylation by 61.7% (**Fig. 2.7C-D**).

3.7. AdipoRon inhibits basal and PDGF-induced ERK phosphorylation in a transient manner in VSMCs

Inhibition of mTOR signaling has also been shown to result in ERK activation through a feedback regulatory mechanism [27, 28]. To determine if AdipoRon inhibition of mTOR signaling has an effect on ERK phosphorylation, AdipoRon-treated (3 hr or 48 hr) VSMCs were stimulated with PDGF for 6 min. **Fig. 2.8A-B** shows that in AdipoRon-treated (25 μM, 3 hr) VSMCs, there was a significant decrease in PDGF-induced ERK phosphorylation by 44%. Upon long-term exposure to AdipoRon (50 μM, 48 hr), there was no significant change in PDGF-induced ERK phosphorylation (**Fig. 2.8C-D**).

To determine the differential effects of AdipoRon on ERK phosphorylation, VSMCs were treated with AdipoRon (25 μM) at increasing time intervals. As shown in **Fig. 2.9A,** AdipoRon treatment resulted in a transient inhibition of ERK phosphorylation at earlier time points (by ~50%, ~60% and ~60% at 6 min, 20 min and 1 hr, respectively) followed by restoration of ERK phosphorylation at the later time points (24 hr and 48 hr). To examine whether AdipoRon-mediated transient decrease in ERK phosphorylation is reflected by similar changes in upstream kinases (C-Raf and MEK), time dependency studies were performed to determine the

phosphorylation state of C-Raf and MEK. Unlike the findings observed with ERK phosphorylation, AdipoRon treatment did not result in significant changes in the phosphorylation of C-Raf and MEK at any of the indicated time points (**Fig. 2.9B**).

3.8. AdipoRon inhibits PDGF receptor tyrosine phosphorylation and its association with p85 adapter subunit of PI 3-kinase in VSMCs

Previous studies have shown that adiponectin inhibits PDGF receptor-β autophosphorylation with an accompanying decrease in ERK phosphorylation [29]. In the present study, exposure of VSMCs to AdipoRon at 25 μM concentration led to a significant decrease in PDGF-induced phosphorylation of $PDGFR^{Tyr751}$ by ~53.4%, as revealed by immunoblot analysis (**Fig. 2.10A** and **B**). In addition, immunoprecipitation studies revealed a significant decrease in the association of PDGF receptor with the p85 adapter subunit of PI 3-kinase by 47% (**Fig. 2.10C**).

3.9. AdipoRon attenuates S6 phosphorylation in the neointimal layer of injured femoral artery

Since AdipoRon inhibited mTOR/p70S6K signaling in VSMCs independent of AMPK (as illustrated in **Fig. 2.6**), we next examined the phosphorylation state of S6 ribosomal protein (a downstream target of mTOR/p70S6K signaling) in the injured femoral artery. Immunohistochemical analysis revealed a significant decrease in S6 phosphorylation in the neointimal layer in AdipoRon-treated group, compared with control (**Fig. 2.11A and B**). In conjunction with the observed findings from VSMCs *in vitro*, these data suggest that the decrease in mTOR/p70S6K signaling may contribute in part to AdipoRon inhibition of neointima formation in the injured artery.

Discussion

The present findings reveal that oral administration of AdipoRon, a small-molecule adiponectin receptor agonist, attenuates neointima formation in the injured femoral artery in C57BL/6J mice. Under *in vitro* conditions, AdipoRon inhibits PDGF-induced VSMC proliferation through mechanisms involving AMPK-independent inhibition of mTOR/p70S6K/S6 signaling (**Fig. 2.12**). In particular, AdipoRon induces a rapid and sustained phosphorylation of AMPK and its downstream target, ACC. In addition, AdipoRon inhibits PDGF-induced phosphorylation of mTOR and its downstream targets, including p70S6K/S6 and 4E-BP1. These inhibitory effects on the phosphorylation of p70S6K/S6 and 4E-BP1 signaling components are persistent even after target-specific downregulation of endogenous AMPK using siRNA, suggesting AMPK-independent effects for AdipoRon inhibition of PDGF-induced mTOR signaling and cyclin D1 expression in VSMCs. Furthermore, AdipoRon treatment results in a sustained and transient decrease in PDGF-induced phosphorylation of Akt and ERK, respectively. Importantly, PDGF receptor-□ tyrosine phosphorylation, which controls the phosphorylation state of Akt and ERK, is diminished upon AdipoRon treatment. Thus, AdipoRon has the potential to inhibit key proliferative signaling events, including mTOR/p70S6K, induced in response to PDGF, a potent mitogen released at the site of arterial injury [30, 31].

In conjunction with several previous reports that document the ability of adiponectin to inhibit PDGF-induced VSMC proliferation [6, 16-18], the present findings suggest that AdipoRon may provide a novel treatment option to attenuate exaggerated VSMC proliferation. From a mechanistic standpoint, adiponectin has been shown to have multiple targets that contribute to inhibition of VSMC proliferation. First, full length adiponectin binds with PDGF-

BB (a potent VSMC mitogen), thereby inhibiting its association with PDGF receptor in VSMCs [17]. In particular, high molecular weight and medium molecular weight forms of adiponectin bind with PDGF-BB, thereby precluding its bioavailability at the pre-receptor level [18]. Second, full length adiponectin has been shown to inhibit autophosphorylation of PDGF receptor-β and the activation of its downstream effector, ERK [17]. Third, adiponectin inhibition of PDGF-induced VSMC proliferation does not require adiponectin receptors (AdipoR1 and/or AdipoR2), as evidenced in studies involving target-specific downregulation of AdipoR1/R2 [18]. Fourth, treatment of VSMCs with adiponectin leads to the activation of AMPK [13, 32], which is implicated in the suppression of VSMC proliferation [14, 15, 33]. Although AdipoRon activates AMPK in a robust and sustained manner in VSMCs, it inhibits PDGF-induced VSMC proliferation through an AMPK-independent mechanism.

It is noteworthy that both adiponectin and AdipoRon promote AMPK activation in VSMCs. Yet, they exhibit differences in the temporal activation of AMPK. For instance, recombinant full-length adiponectin has been shown to enhance $AMPK^{Thr172}$ phosphorylation at 30 min and 6-12 hr time points in rat aortic VSMCs [34] and human aortic VSMCs [32], respectively. In a different study, treatment with full-length adiponectin enriched in HMW oligomers, but not truncated globular adiponectin, leads to a detectable increase in $AMPK^{Thr172}$ phosphorylation at the 24 time point in human coronary artery VSMCs [13]. Regardless of the monomeric or oligomeric forms, adiponectin requires a much longer time frame to enhance $AMPK^{Thr172}$ phosphorylation in VSMCs. In contrast to adiponectin, AdipoRon enhances $AMPK^{Thr172}$ phosphorylation as early as 6 min, which remains elevated in a sustained manner for up to 48 hr in human aortic VSMCs (present study). These findings are consistent with the recent observations of a rapid rise in $AMPK^{Thr172}$ phosphorylation in AdipoRon-treated mouse myoblast

C2C12 cell line [9]. As expected, the increase in AMPK^{Thr172} phosphorylation by adiponectin or AdipoRon results in enhanced phosphorylation of ACC (a downstream target of AMPK). It is likely that the differences in the temporal activation of AMPK by adiponectin and AdipoRon are attributable to the differential activation of proximal signaling events, including adipoR1/R2 and LKB1 [35], in VSMCs.

Studies by several investigators, including our recent findings, have shown that AMPK activation results in the suppression of mTOR/p70S6K signaling in different tissues/cell types [36, 37], including VSMCs [14, 15]. Importantly, adiponectin- or AdipoRon-mediated AMPK activation is associated with inhibition of mTOR/p70S6K signaling in VSMCs. Yet, there are differences in the cause and effect relationship between these two signaling events depending on the agonist. For instance, adiponectin-mediated AMPK activation leads to inhibition of mTOR/p70S6K signaling in human coronary or femoral artery VSMCs, as evidenced in experimental approaches involving the use of pharmacological agents (e.g., compound C, an AMPK inhibitor) or target-specific siRNAs [13, 38]. In the present study involving AMPK downregulation by target-specific siRNA, AdipoRon-mediated decrease in mTOR signaling remains essentially unaltered. This is revealed by persistent decreases in the phosphorylation state of p70S6K/S6 and 4E-BP1 signaling components under basal and PDGF-stimulated conditions. Taken together, although AMPK activation by adiponectin is critical for the suppression of mTOR/p70S6K signaling [13, 38], AdipoRon-mediated inhibition of mTOR/p70S6K signaling occurs independent of AMPK activation in VSMCs.

Previous studies have documented the existence of a negative feedback regulatory loop whereby inhibition of mTOR/p70S6K signaling results in the activation of Akt in different cell types under normal and diseased states [26-28]. Notably, adiponectin-mediated inhibition of

mTOR/p70S6K signaling is associated with Akt activation (at 4-24 hour time points) in human coronary artery VSMCs [13]. In addition, adiponectin overexpression diminishes p70S6K signaling with an accompanying increase in Akt phosphorylation in VSMCs [39]. However, in a different study, adiponectin does not affect Akt phosphorylation in human VSMCs [32]. Furthermore, C1q/TNF-related protein-9 (CTRP9), an adipocytokine and a conserved paralog of adiponectin, fails to activate Akt in human VSMCs [40]. In the present study, acute or prolonged treatment with AdipoRon leads to inhibition of PDGF-induced Akt phosphorylation in VSMCs. Together, in the absence of an apparent negative feedback regulatory loop, AdipoRon treatment may inhibit PDGF receptor-mediated proximal signaling components, including Akt in VSMCs.

Although inhibition of mTOR/p70S6K or PI 3-kinase may lead to ERK inhibition [41] or ERK activation [26-28] in different cell types, studies with adiponectin demonstrate its inhibitory effects on PDGF-induced ERK phosphorylation in human VSMCs [17]. In addition, adiponectin attenuates IGF1-induced ERK phosphorylation in rat aortic VSMCs [34]. However, in a different study, adiponectin induces ERK phosphorylation in porcine coronary artery VSMCs [42]. In the present study, AdipoRon treatment between 6 min and 48 hr does not affect the basal phosphorylation of C-Raf and MEK, the kinases upstream of ERK. However, AdipoRon treatment results in a transient decrease in basal ERK phosphorylation at 6-60 min time points with a reversal to the pre-existing phosphorylation state at 24-48 hr time points. Accordingly, acute exposure to AdipoRon inhibits PDGF-induced ERK phosphorylation, whereas prolonged treatment with AdipoRon does not result in significant changes in PDGF-induced ERK phosphorylation. At this juncture, it is important to note that a transient decrease in ERK phosphorylation followed by its reactivation has been evidenced in recent studies with a PI 3-

kinase inhibitor [27, 28]. Further studies are clearly warranted to determine AdipoRon regulation of PI 3-kinase activity and its relationship with C-Raf/MEK-independent ERK signaling [43, 44] in VSMCs.

Furthermore, AdipoRon inhibits PDGF receptor-β tyrosine phosphorylation in VSMCs, as has been evidenced in previous studies with adiponectin [17]. Since adiponectin inhibits PDGF ligand association with PDGF receptor [17, 18] and activates protein tyrosine phosphatase 1B [45], future studies should determine which of these two mechanisms mediates AdipoRon inhibition of PDGF receptor-β tyrosine phosphorylation. Importantly, this decrease in PDGF receptor tyrosine phosphorylation may contribute in part to the observed inhibitory effects of AdipoRon on: i) the association of p85 (adapter subunit of PI 3-kinase) with the activated PDGF receptor; ii) Akt activation; and iii) mTOR/p70S6K/S6 and 4E-BP1 signaling in VSMCs.

In conclusion, the present findings strongly suggest that orally-administered AdipoRon has the potential to limit restenosis after angioplasty at the lesion site by targeting VSMC proliferative signaling events, including mTOR/p70S6K. Although AdipoRon activates AMPK reminiscent of adiponectin action, AMPK activation does not play an intermediary role toward AdipoRon-mediated inhibition of VSMC proliferation. In a recent study, AdipoRon has been shown to promote vascular smooth muscle relaxation through AMPK-independent mechanism [25]. In view of the reported beneficial effects of AdipoRon in improving glycemic control in type 2 diabetic mice [9], strategies that utilize this small-molecule to suppress exaggerated VSMC proliferation may provide a realistic alternative to rapamycin/sirolimus (an mTOR inhibitor), which has been shown to exhibit adverse effects including glucose intolerance and diabetes in rodent models [46, 47].

Acknowledgements

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Conflict of Interest

The authors declare no conflicts of interest.

Figure legends

Fig. 2.1. Effects of AdipoRon on injury-induced neointima formation in mouse femoral artery. AdipoRon was administered orally (50 mg/Kg) a day before femoral artery injury and for the following 21 days until sacrifice. Femoral artery sections from AdipoRon- and vehicle-treated (Control) mice were then subjected to: **A**) Hematoxylin and Eosin (H&E); and **B**) Elastic Van Gieson (EVG) staining. The arrows indicate internal and external elastic laminae; scale bars represent 100 μm. **C**) Morphometric analyses of injured femoral arteries that include intima/media ratio and intimal area. The data shown are the means ± SEM. * $p < 0.05$; n = 5 to 7 mice/group.

Fig. 2.1A-C

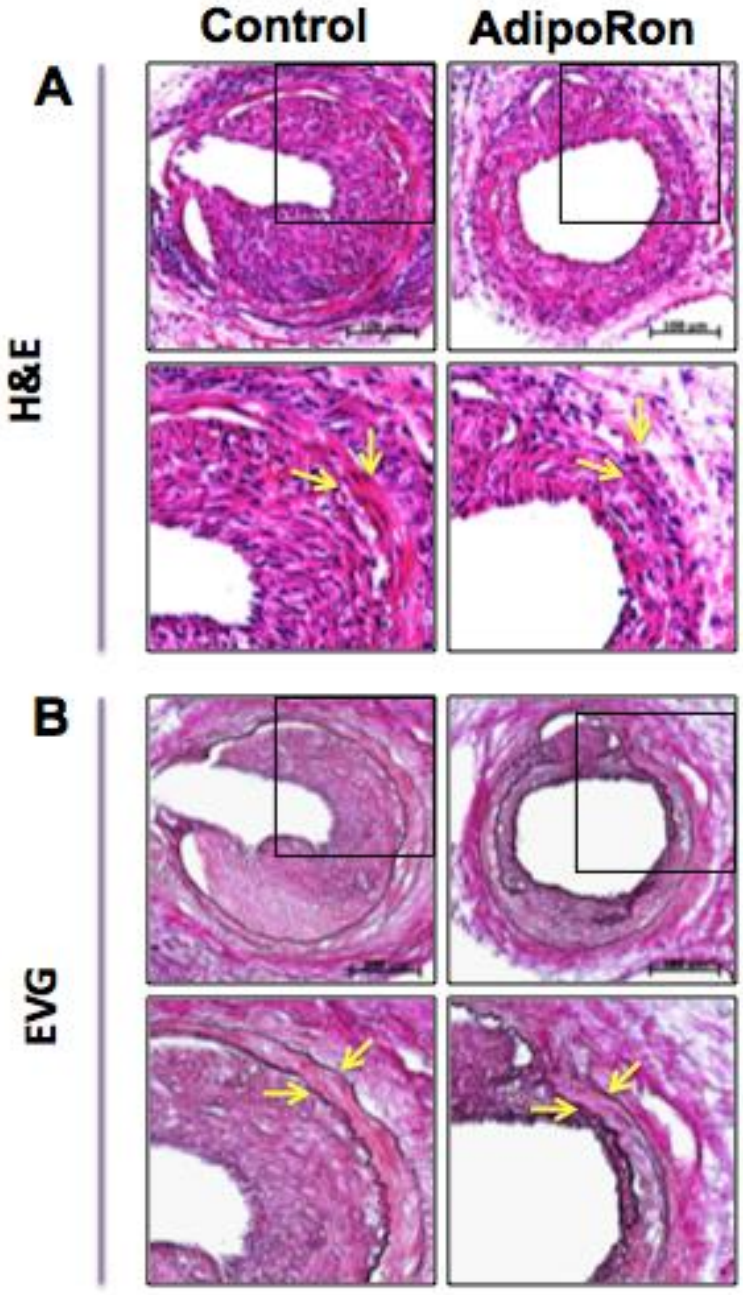
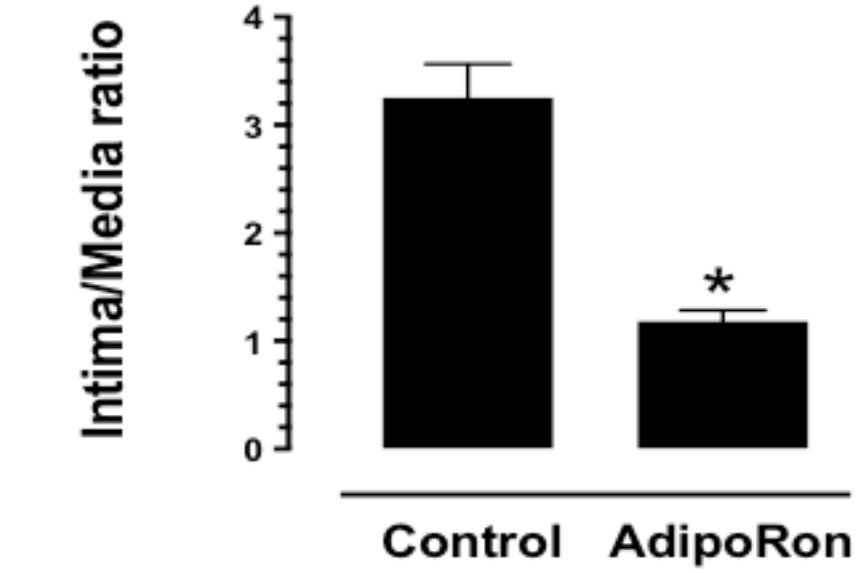


Fig. 2.1A-C (contd.)

C



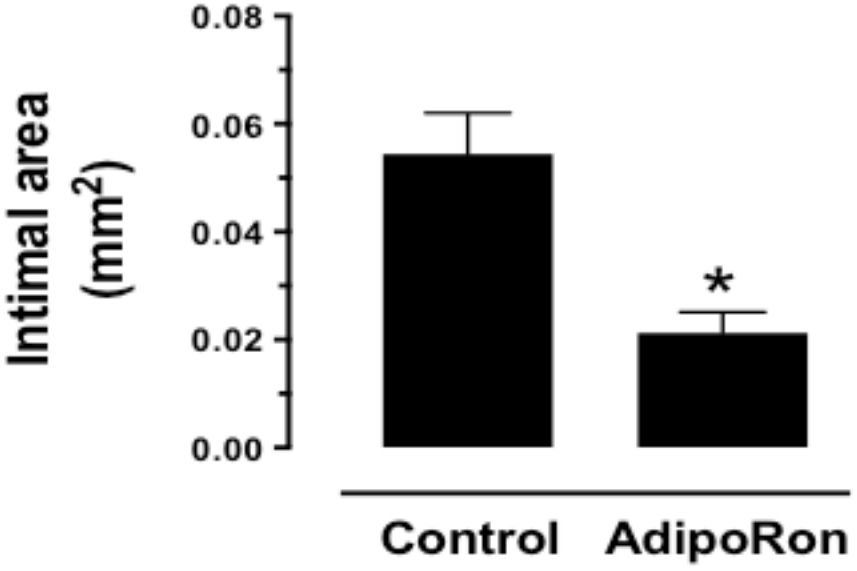


Fig. 2.2. Effects of AdipoRon on smooth muscle α-actin and Ki-67 immunoreactivity in the injured femoral artery. Confocal immunofluorescence analyses show the images for **A)** smooth muscle (SM) α-actin and **B)** Ki-67 (in red). The representative images for nuclei (DAPI, blue), elastin autofluorescence (laminae, green), and merged staining are also shown. The arrows indicate internal and external elastic laminae; scale bars represent 100 μm.

Fig. 2.2A-B

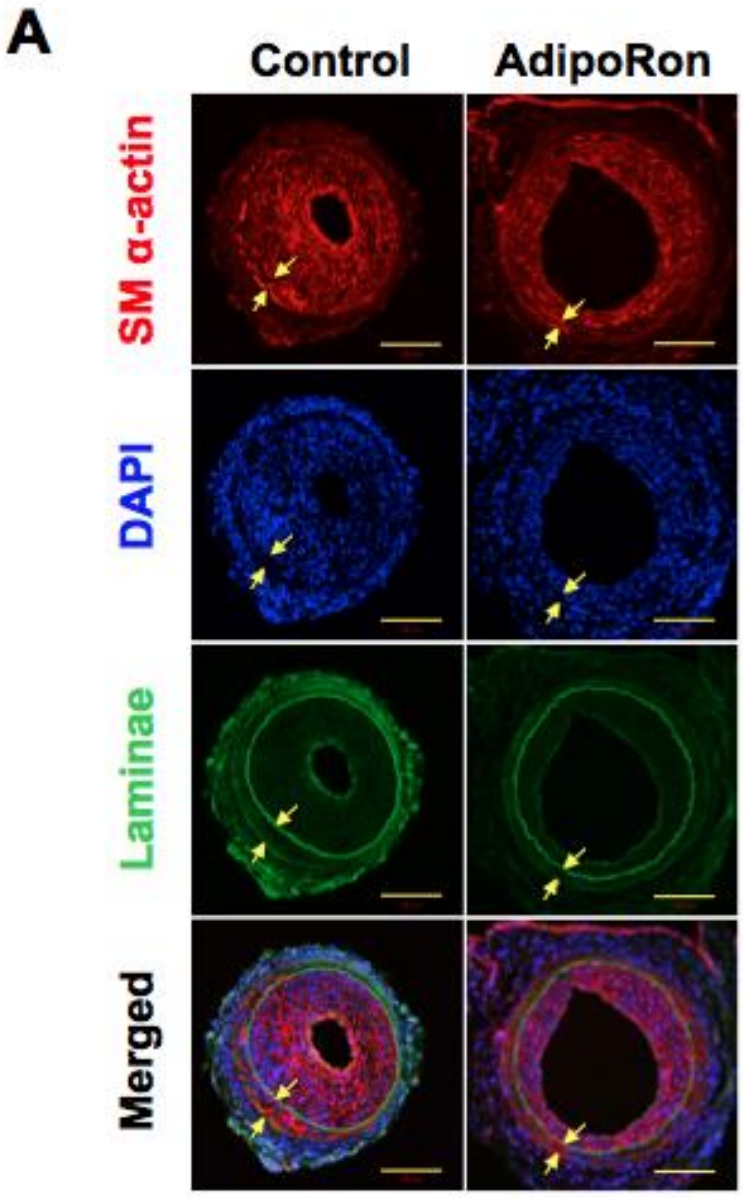


Fig. 2.2A-B
(contd.)

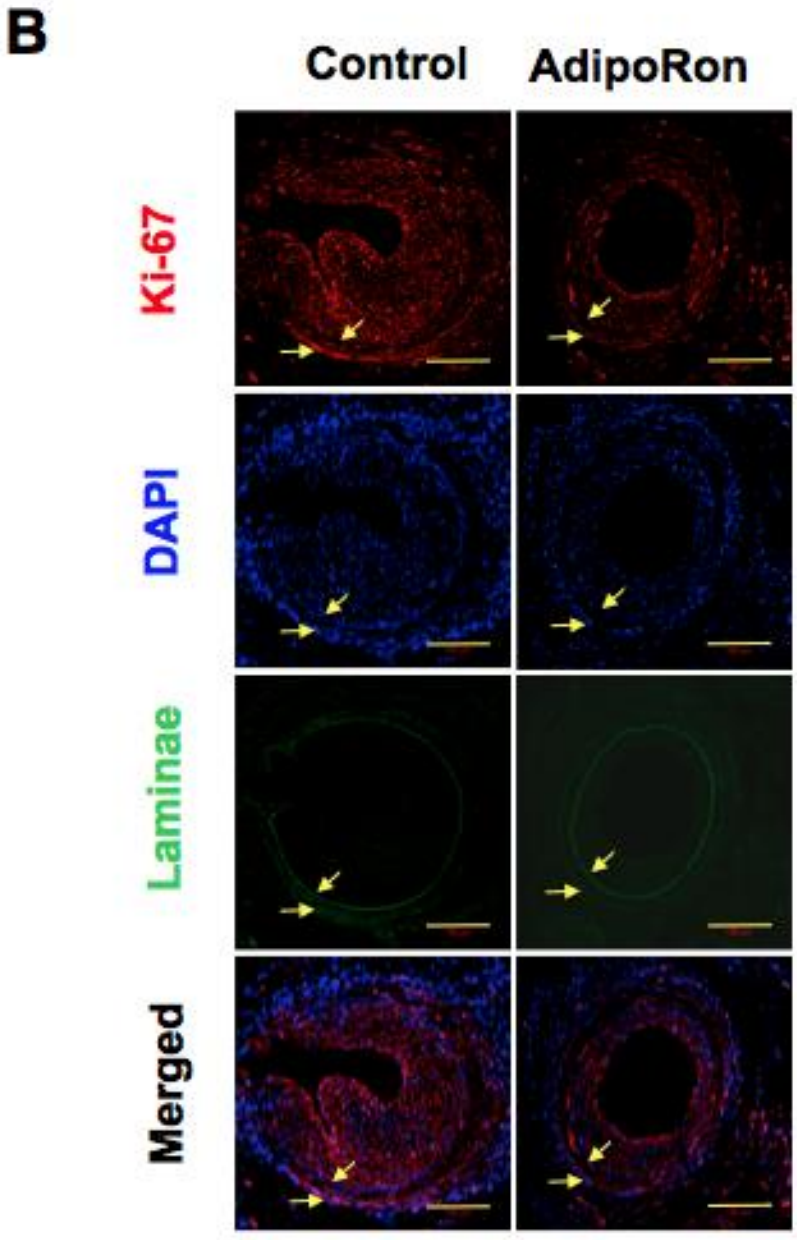


Fig. 2.3. Effects of AdipoRon on basal and PDGF-induced VSMC proliferation. Serum-deprived VSMCs were incubated with: **A)** increasing concentrations of AdipoRon (5 to 100 μM) for 96 hr to determine the changes in Alamar blue fluorescence (n = 8); **B)** AdipoRon (5 to 100 μM) for 30 min followed by exposure to PDGF (30 ng/ml) for 96 hr to determine the changes in cell counts (n = 5); **C)** AdipoRon (50 μM) for 30 min followed by exposure to PDGF (30 ng/ml) for 24 hr to determine the changes in DNA synthesis (n=3); and **D)** AdipoRon (25 μM) for 30 min followed by exposure to PDGF (30 ng/ml) for 48 hr to determine the changes in cyclin D1 expression (n = 5). □-actin was used as an internal control. The data shown are the means ± SEM. *, # $p < 0.05$ compared with control (- PDGF and/or 0 μM AdipoRon) or PDGF (+ PDGF and 0 μM AdipoRon), respectively, using one-way ANOVA (A) or two-way ANOVA (B, C and D) followed by Bonferroni multiple comparisons test.

Fig. 2.3A-D

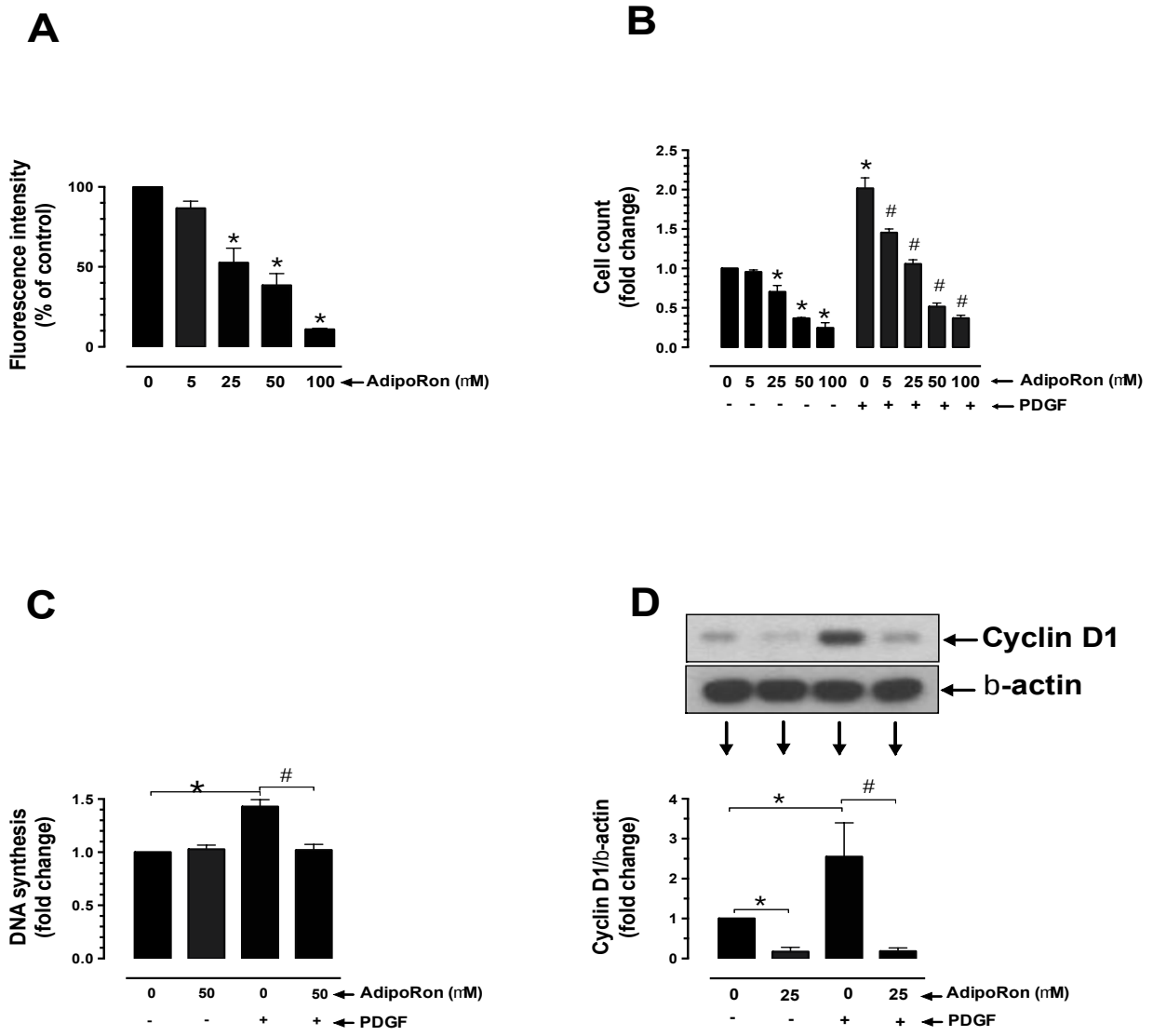

A
Fluorescence intensity (% of control)
100
50
0
0 5 25 50 100 ← AdipoRon (mM)
B
Cell count (fold change)
2.5
2.0
1.5
1.0
0.5
0.0
0 5 25 50 100 0 5 25 50 100 ← AdipoRon (mM)
- - - - - + + + + + ← PDGF
C
DNA synthesis (fold change)
1.5
1.0
0.5
0.0
0 50 0 50 ← AdipoRon (mM)
- - + + ← PDGF
D
← Cyclin D1
← b-actin
Cyclin D1/b-actin (fold change)
4
3
2
1
0
0 25 0 25 ← AdipoRon (mM)
- - + + ← PDGF

Fig. 2.4. Concentration- and time dependent- effects of AdipoRon on the phosphorylation of AMPK, ACC, and S6 in VSMCs. Serum-deprived VSMCs were incubated with: **A-B)** increasing concentrations of AdipoRon (5 to 100 μM) for 48 hr; or **C-D)** a fixed concentration of AdipoRon (25 μM) at the indicated time intervals. VSMC lysates were then subjected to immunoblot analysis using primary antibodies specific for $pAMPK^{Thr172}$, AMPK, pACC, ACC, pS6 and S6. β-actin was used as internal control. The data shown in the bar graphs are the means ± SEM. * $p < 0.05$ compared with control (0 μM AdipoRon) using one-way ANOVA (B) or repeated measures one-way ANOVA (D) followed by Bonferroni multiple comparisons test (n = 3-5).

Fig. 2.4A-D

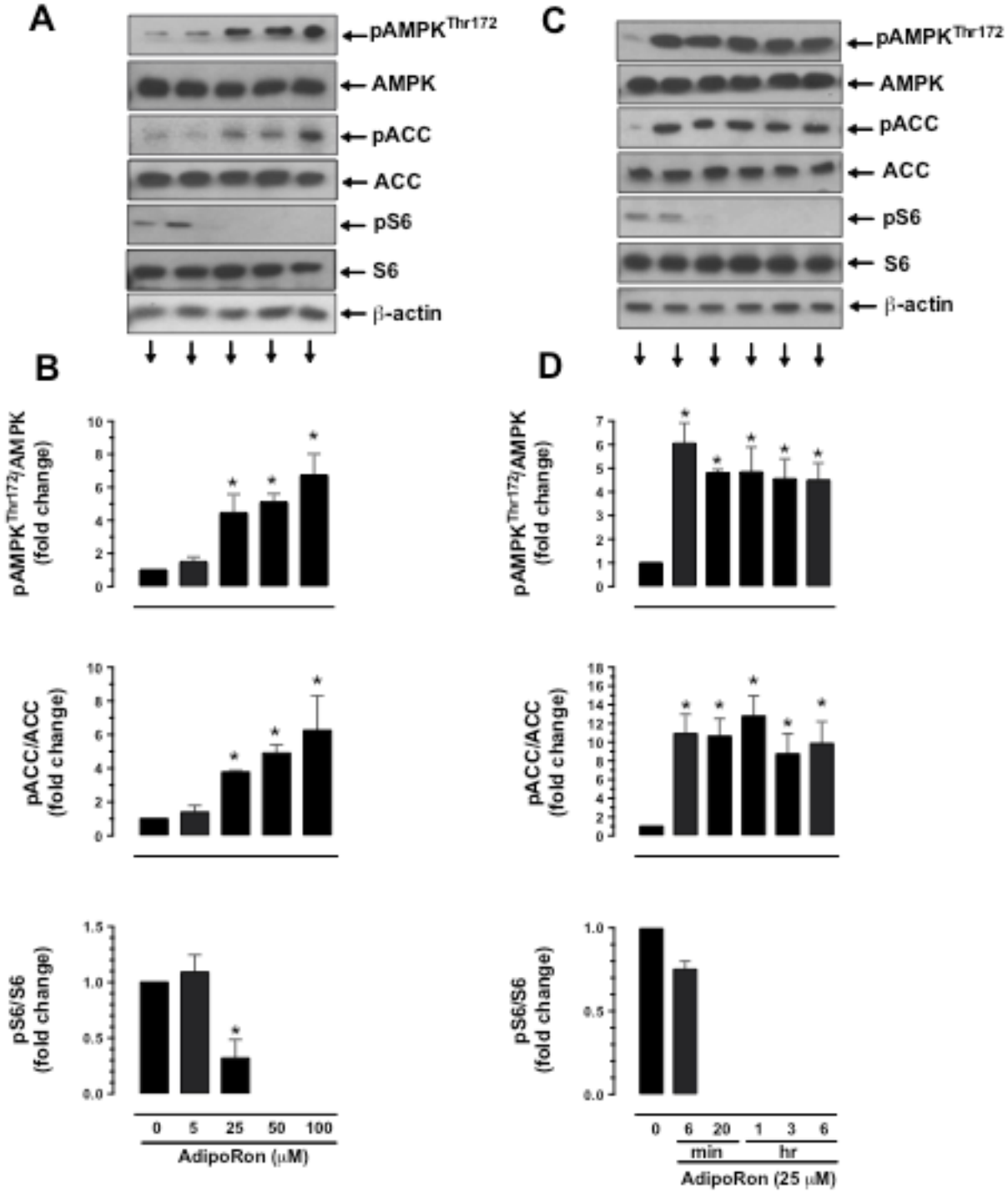

A
pAMPK^Thr172
AMPK
pACC
ACC
pS6
S6
β-actin
C
pAMPK^Thr172
AMPK
pACC
ACC
pS6
S6
β-actin
B
pAMPK^Thr172/AMPK (fold change)
pACC/ACC (fold change)
pS6/S6 (fold change)
0 5 25 50 100
AdipoRon (μM)
D
pAMPK^Thr172/AMPK (fold change)
pACC/ACC (fold change)
pS6/S6 (fold change)
0 6 20 1 3 6
min hr
AdipoRon (25 μM)

Fig. 2.5. Effects of AdipoRon on basal and PDGF-induced phosphorylation of mTOR, p70S6K, S6, and 4E-BP1 in VSMCs. **A-B)** Serum-deprived VSMCs were incubated with AdipoRon (25 μM) for 3 hr followed by stimulation with PDGF (30 ng/ml) for 6 min. VSMC lysates were subjected to immunoblot analysis using primary antibodies specific for $pmTOR^{Ser2448}$, mTOR, pp70S6K, p70S6K, pS6, S6, p4E-BP1 and 4E-BP1. GAPDH was used as an internal control. The data shown in the bar graphs are the means ± SEM. *, # $p < 0.05$ compared with control (- PDGF and 0 μM AdipoRon) or PDGF (+ PDGF and 0 μM AdipoRon), respectively, using two-way ANOVA followed by Bonferroni multiple comparisons test (n = 3).

Fig. 2.5A-B

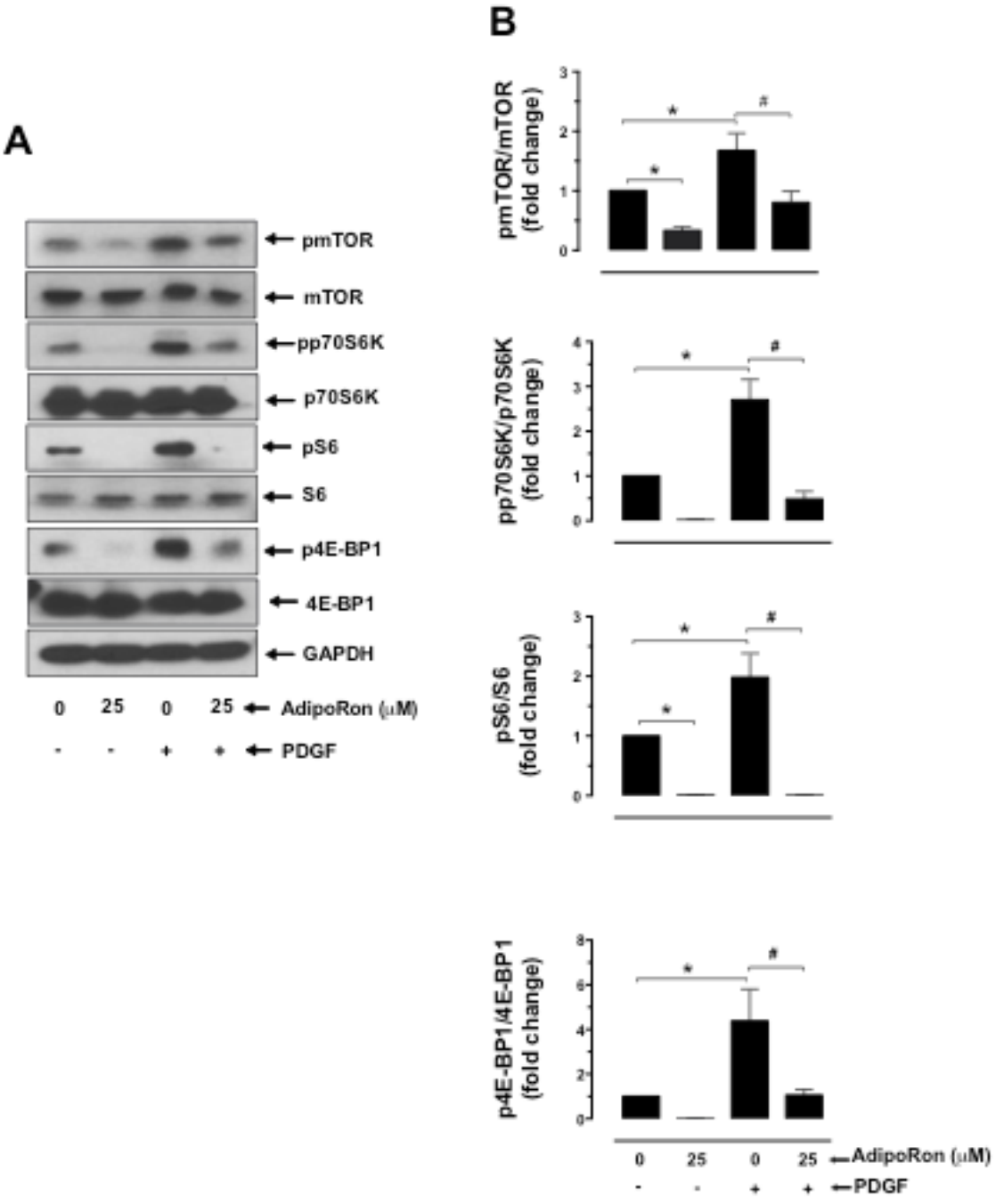

A
pmTOR
mTOR
pp70S6K
p70S6K
pS6
S6
p4E-BP1
4E-BP1
GAPDH
0 25 0 25 AdipoRon (μM)
- - + + PDGF
B
pmTOR/mTOR (fold change)
pp70S6K/p70S6K (fold change)
pS6/S6 (fold change)
p4E-BP1/4E-BP1 (fold change)
0 25 0 25 AdipoRon (μM)
- - + + PDGF

Fig. 2.6. Effects of AMPKα1 downregulation on AdipoRon-mediated changes in basal and PDGF-induced mTOR signaling and cyclin D1 expression in VSMCs. VSMCs were transfected with scrambled (Scr.) or AMPKα1 siRNA and maintained in culture for 48 hr. Subsequently, serum-deprived VSMCs were incubated with **A-C**) AdipoRon (25 μM) for 3 hr followed by stimulation with PDGF (30 ng/ml) for 6 min; or **D)** Adiporon (25 μM) for 30 min followed by exposure to PDGF (30 ng/ml) for 48 hr. VSMC lysates were then subjected to immunoblot analysis using the primary antibodies specific for AMPKα1, pp70S6K, p70S6K, pS6, S6, p4E-BP1, 4E-BP1 and cyclin D1. β-actin was used as internal control. The data shown in the bar graphs are the means ± SEM. * $p < 0.05$ compared with control (- PDGF and 0 μM AdipoRon) [C and D] or Scr. siRNA (B). # $p < 0.05$ compared with PDGF (+ PDGF and 0 μM AdipoRon) [C and D]. Statistical significance was determined by applying unpaired student t test (B) or two-way ANOVA followed by Bonferroni multiple comparisons test (C and D) (n = 3).

Fig. 2.6A-D

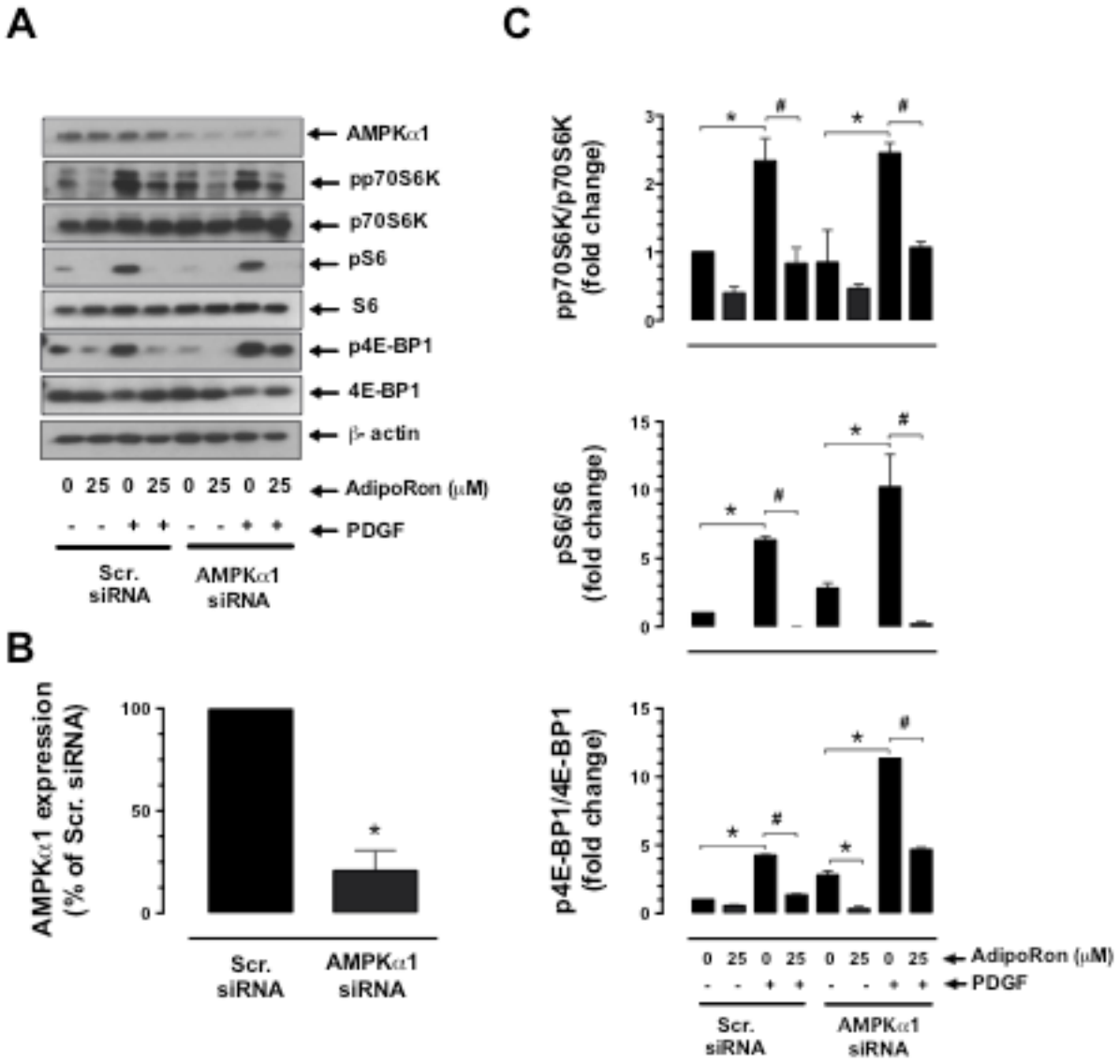

A
AMPKα1
pp70S6K
p70S6K
pS6
S6
p4E-BP1
4E-BP1
β- actin
0 25 0 25 0 25 0 25
AdipoRon (μM)
- - + + - - + +
PDGF
Scr. siRNA
AMPKα1 siRNA
B
AMPKα1 expression (% of Scr. siRNA)
100
50
0
*
Scr. siRNA
AMPKα1 siRNA
C
pp70S6K/p70S6K (fold change)
pS6/S6 (fold change)
p4E-BP1/4E-BP1 (fold change)
0 25 0 25 0 25 0 25
AdipoRon (μM)
- - + + - - + +
PDGF
Scr. siRNA
AMPKα1 siRNA

Fig. 2.6A-D (contd.)

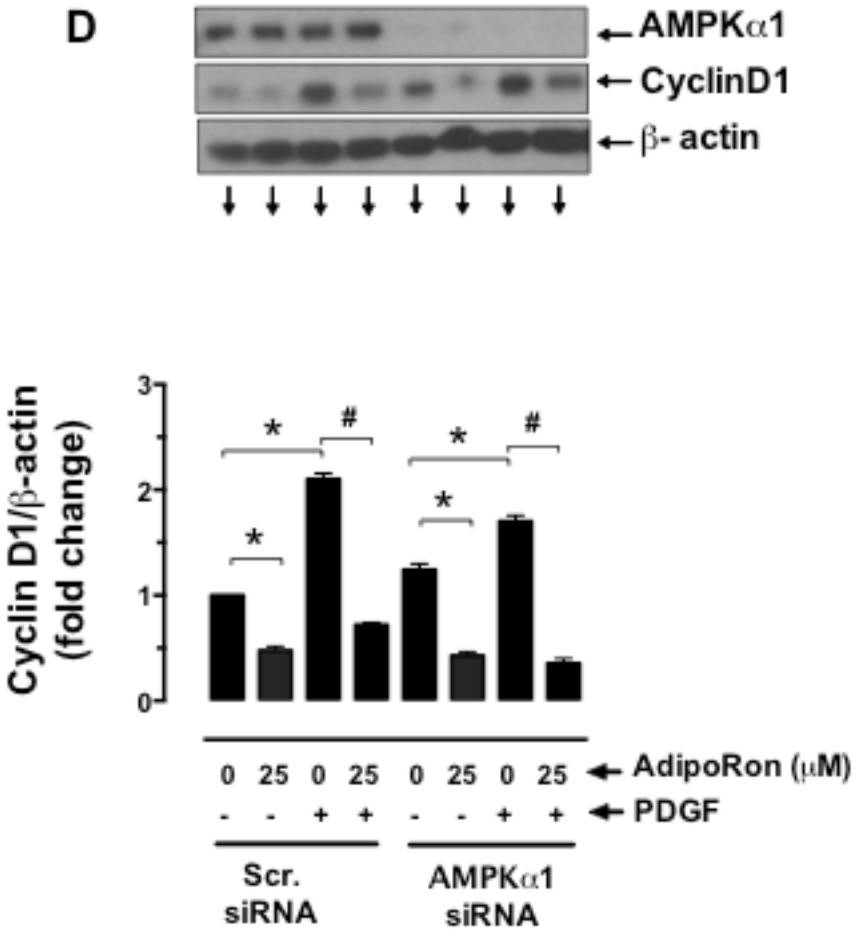


Fig. 2.7. Effects of AdipoRon on basal and PDGF-induced phosphorylation of Akt in VSMCs. Serum-deprived VSMCs were incubated with: **A-B**) AdipoRon (25 μM) for 3 hr or **C-D**) AdipoRon (50 μM) for 48 hr followed by stimulation with PDGF (30 ng/ml) for 6 min. VSMC lysates were subjected to immunoblot analysis using primary antibody specific for $pAkt^{Ser473}$, $pAkt^{Thr308}$ and Akt. □-actin was used as an internal control. The data shown in the bar graphs are the means ± SEM. * $p < 0.05$ using unpaired student t test ($n = 3$).

Fig. 2.7A-D

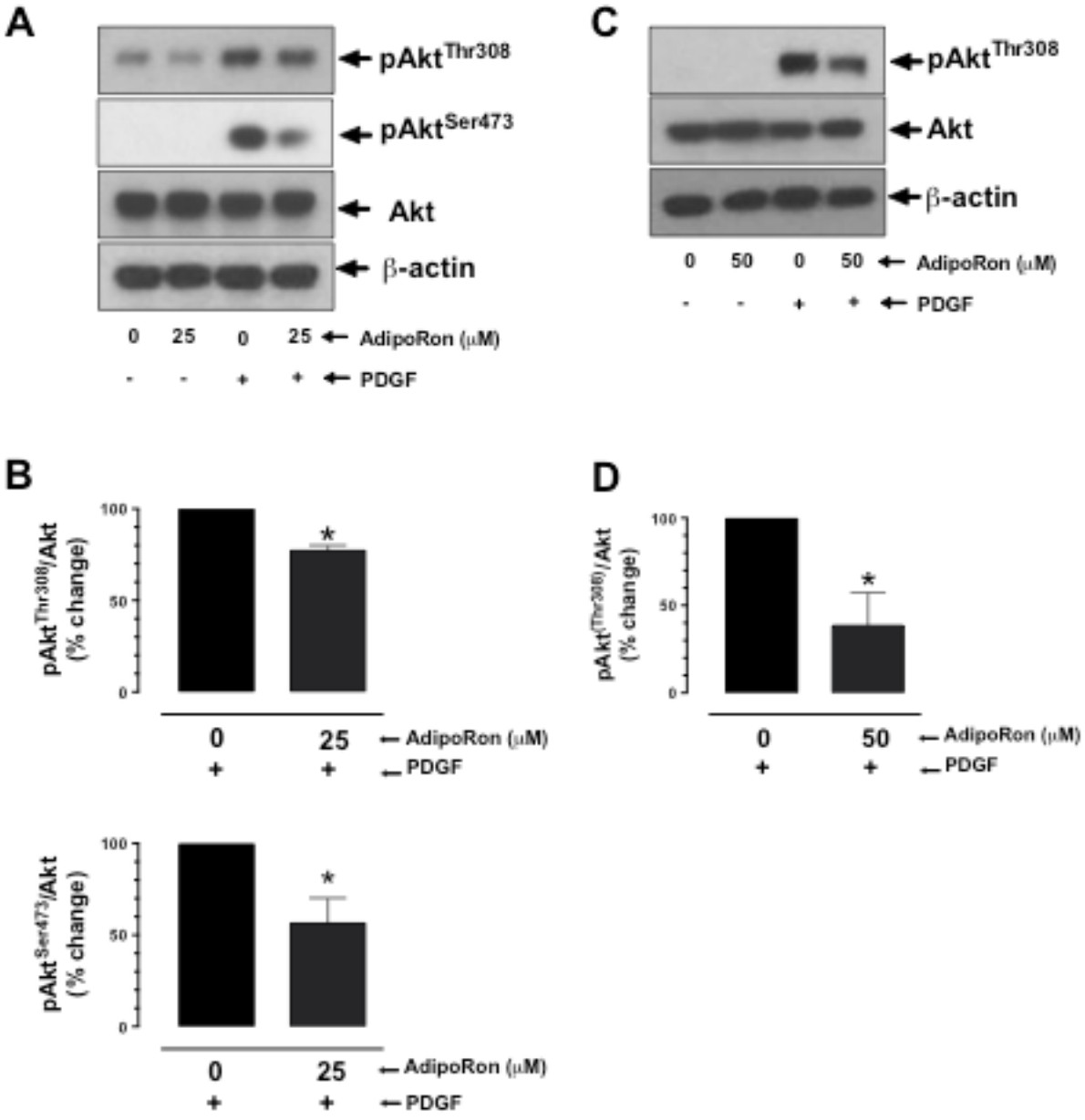

A
pAkt^Thr308
pAkt^Ser473
Akt
β-actin
0 25 0 25 AdipoRon (μM)
- - + + PDGF
C
pAkt^Thr308
Akt
β-actin
0 50 0 50 AdipoRon (μM)
- - + + PDGF
B
pAkt^Thr308/Akt (% change)
100
50
0
*
0 25 AdipoRon (μM)
+ + PDGF
pAkt^Ser473/Akt (% change)
100
50
0
*
0 25 AdipoRon (μM)
+ + PDGF
D
pAkt^(Thr308)/Akt (% change)
100
50
0
*
0 50 AdipoRon (μM)
+ + PDGF

Fig. 2.8. Effects of AdipoRon on basal and PDGF-induced phosphorylation of ERK in VSMCs. Serum-deprived VSMCs were incubated with: **A-B**) AdipoRon (25 μM) for 3 hr or **C-D)** AdipoRon (50 μM) for 48 hr followed by stimulation with PDGF (30 ng/ml) for 6 min. VSMC lysates were subjected to immunoblot analysis using primary antibodies specific for pERK and ERK. GAPDH was used as an internal control. The data shown in the bar graphs are the means ± SEM. * $p < 0.05$ using unpaired student t test; NS, not significant (n = 3).

Fig. 2.8A-D

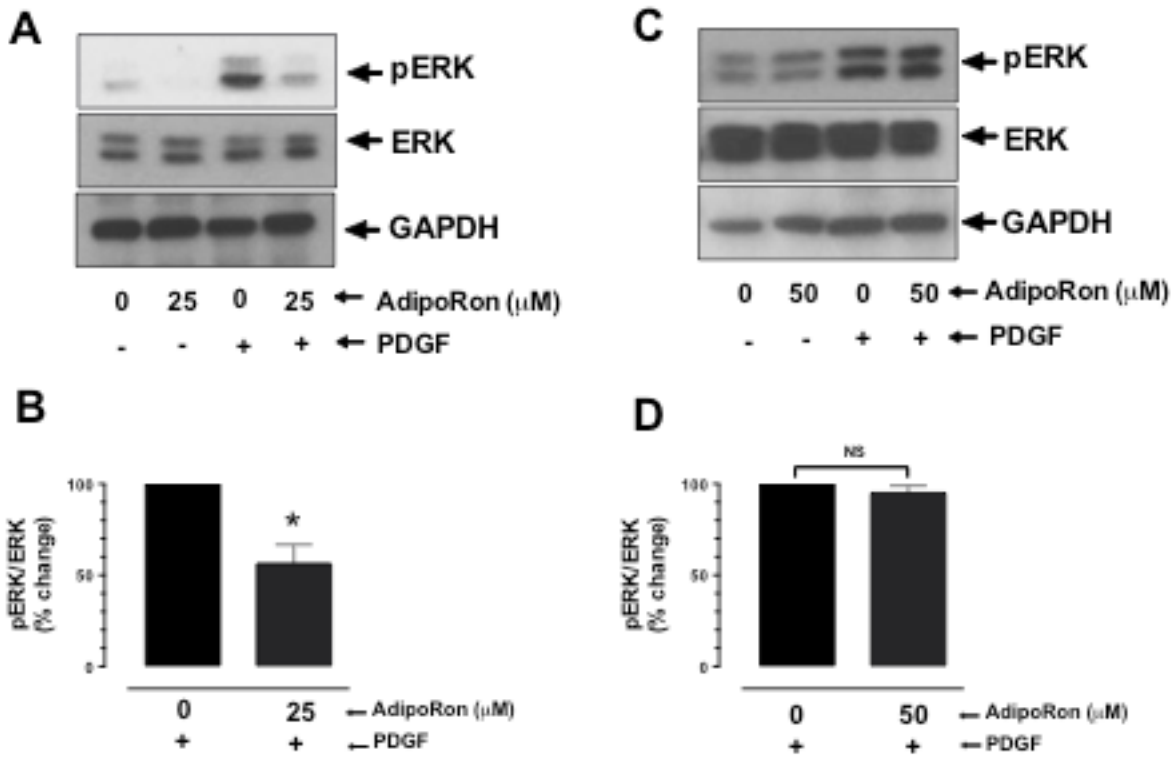

A
pERK
ERK
GAPDH
0 25 0 25 AdipoRon (μM)
- - + + PDGF
C
pERK
ERK
GAPDH
0 50 0 50 AdipoRon (μM)
- - + + PDGF
B
pERK/ERK (% change)
100
50
0
*
0 25 AdipoRon (μM)
+ + PDGF
D
NS
pERK/ERK (% change)
100
50
0
0 50 AdipoRon (μM)
+ + PDGF

Fig. 2.9. Time-dependent effects of AdipoRon on the phosphorylation of ERK, C-Raf, and MEK in VSMCs. Serum-deprived VSMCs were incubated with AdipoRon (25 μM) at increasing time intervals (6 min to 48 hr). VSMC lysates were subjected to immunoblot analysis using primary antibody specific for **A)** pERK and ERK or **B)** pC-Raf and C-Raf or pMEK and MEK. β-actin or GAPDH was used as internal controls. The data shown in the bar graphs are the means ± SEM. * $p < 0.05$ using repeated measures one-way ANOVA followed by Bonferroni multiple comparisons test ($n = 3$).

Fig. 2.9A-B

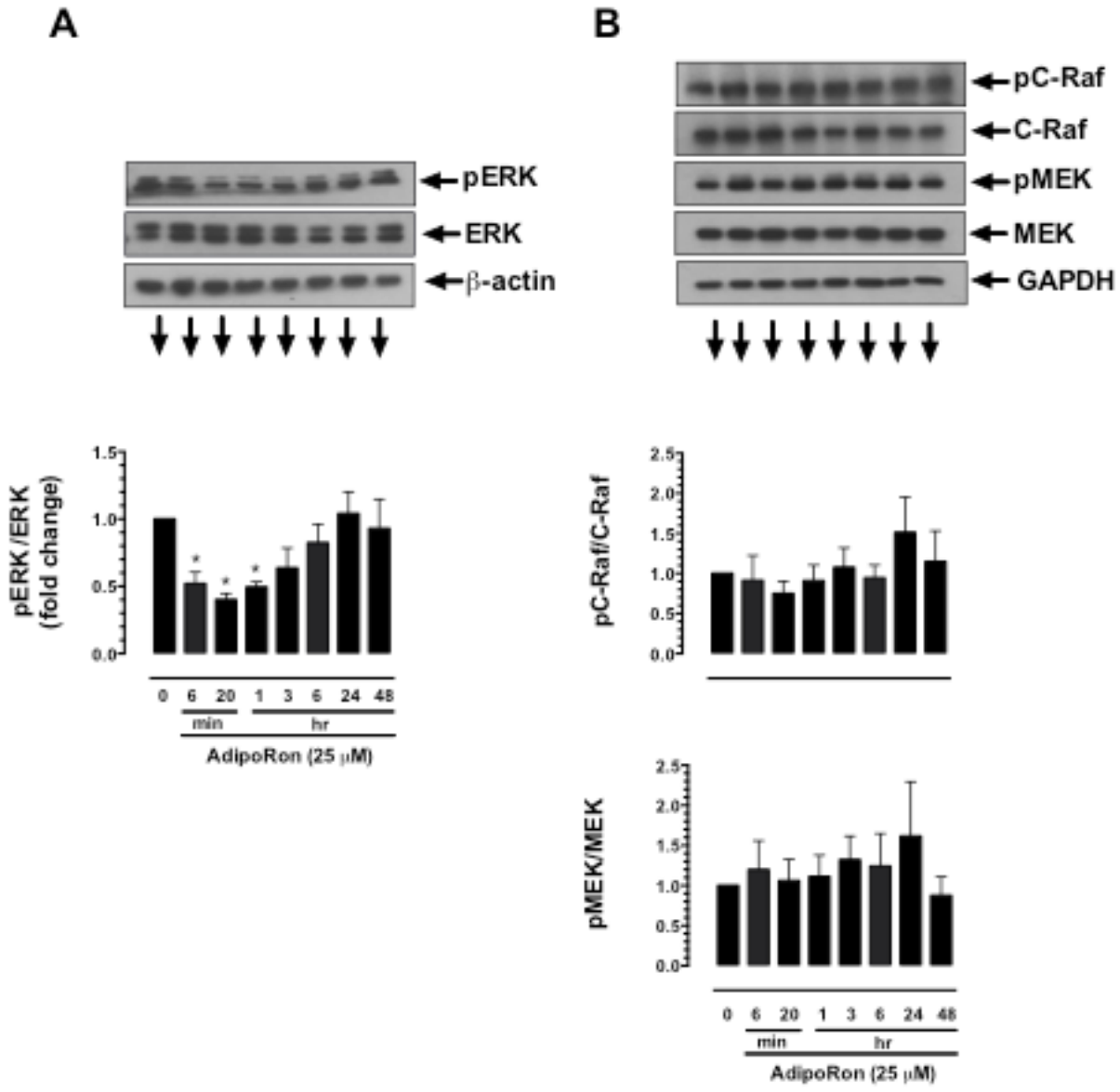

A
pERK
ERK
β-actin
pERK/ERK (fold change)
1.5
1.0
0.5
0.0
0 6 20 1 3 6 24 48
min
hr
AdipoRon (25 μM)
B
pC-Raf
C-Raf
pMEK
MEK
GAPDH
pC-Raf/C-Raf
2.5
2.0
1.5
1.0
0.5
0.0
pMEK/MEK
0 6 20 1 3 6 24 48
min
hr
AdipoRon (25 μM)

Fig. 2.10. Effect of AdipoRon on PDGFR-β tyrosine phosphorylation and its association with p85 adaptor subunit of PI 3-kinase in VSMCs. **A-B)** Serum-deprived VSMCs were incubated with AdipoRon (25 μM) for 3 hr followed by stimulation with PDGF (30 ng/ml) for 6 min. VSMC lysates were then subjected to immunoblot analysis using primary antibodies specific for p-PDGFR-β^{Tyr751} and PDGFR-β. GAPDH was used as internal control. **C)** Serum-deprived VSMCs were incubated with AdipoRon (50 μM) for 48 hr followed by stimulation with PDGF (30 ng/ml) for 2 min. The cell lysates were subjected to immunoprecipitation (IP) using PDGFR-β primary antibody and then probed with p85 primary antibody. The data shown in the bar graphs are the means ± SEM. *, # $p < 0.05$ compared with control (- PDGF and/or 0 μM AdipoRon) or PDGF (+ PDGF and 0 μM AdipoRon), respectively, using unpaired student t test (B) or two-way ANOVA followed by Bonferroni multiple comparisons test (C) [n = 3]. MC = Mock, HC = heavy chain.

Fig. 2.10A-C

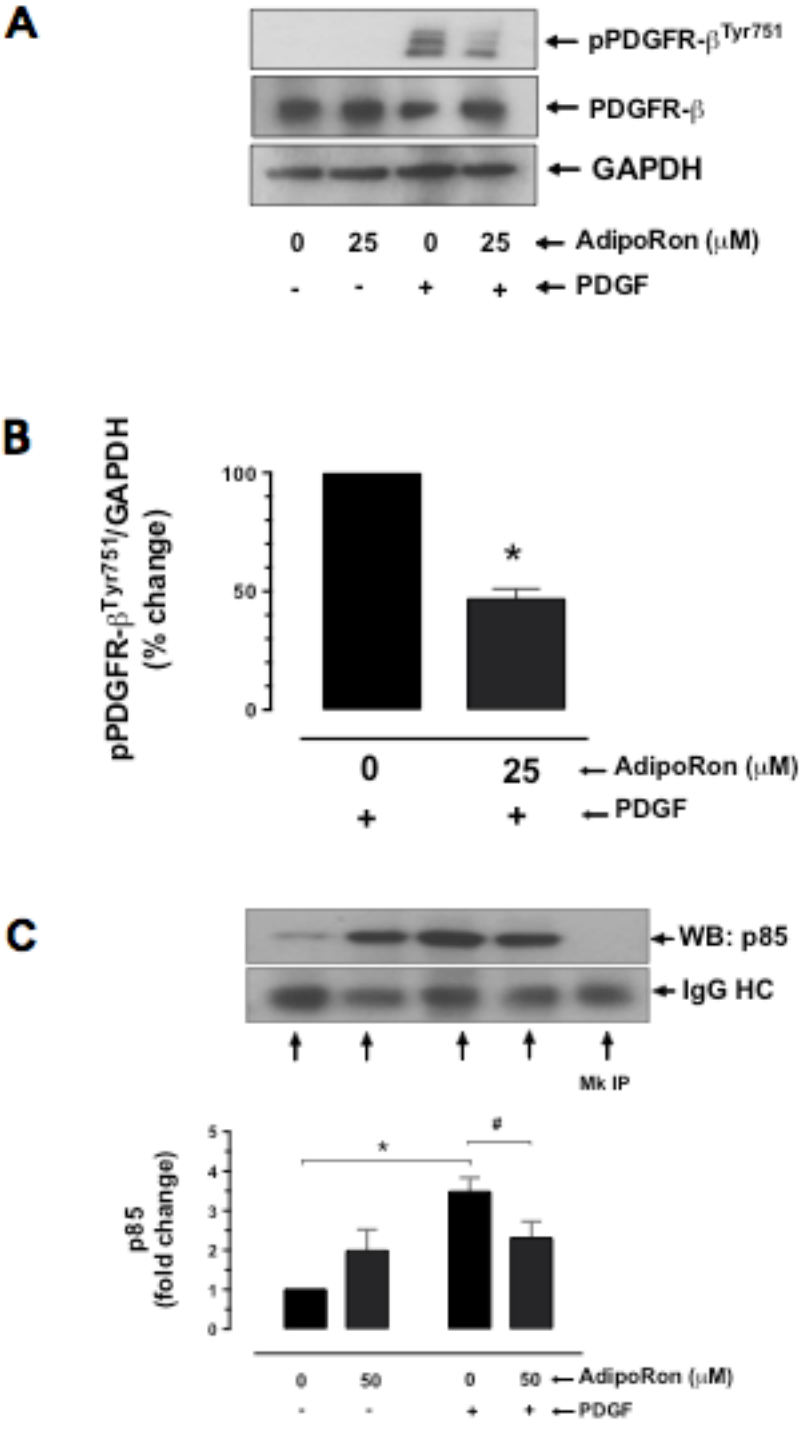

A
pPDGFR-β Tyr751
PDGFR-β
GAPDH
0 25 0 25 AdipoRon (μM)
- - + + PDGF
B
pPDGFR-β Tyr751/GAPDH (% change)
100
50
0
*
0 25 AdipoRon (μM)
+ + PDGF
C
WB: p85
IgG HC
Mk IP
p85 (fold change)
5
4
3
2
1
0
*
#
0 50 0 50 AdipoRon (μM)
- - + + PDGF

Fig. 2.11. Effects of AdipoRon on the phosphorylation state of S6 ribosomal protein in the injury femoral artery. The femoral artery sections from AdipoRon-treated and control mice were subjected to immunohistochemical analysis using primary antibody specific for pS6. **A)** The representative images of pS6 immunoreactivity were visualized using diaminobenzidine (DAB) staining at a magnification 20x. The scale bars represent 100 μm (upper panel). Black arrows indicate internal and external elastic laminae; yellow arrows indicate pS6 (lower panel). **B)** The intensity of pS6 staining was quantified and expressed as reciprocal intensities. The data shown in the bar graph are the means ± SEM. * $p < 0.05$ using unpaired t-test ($n = 3$). a. u. = arbitrary units.

Fig. 2.11A-B

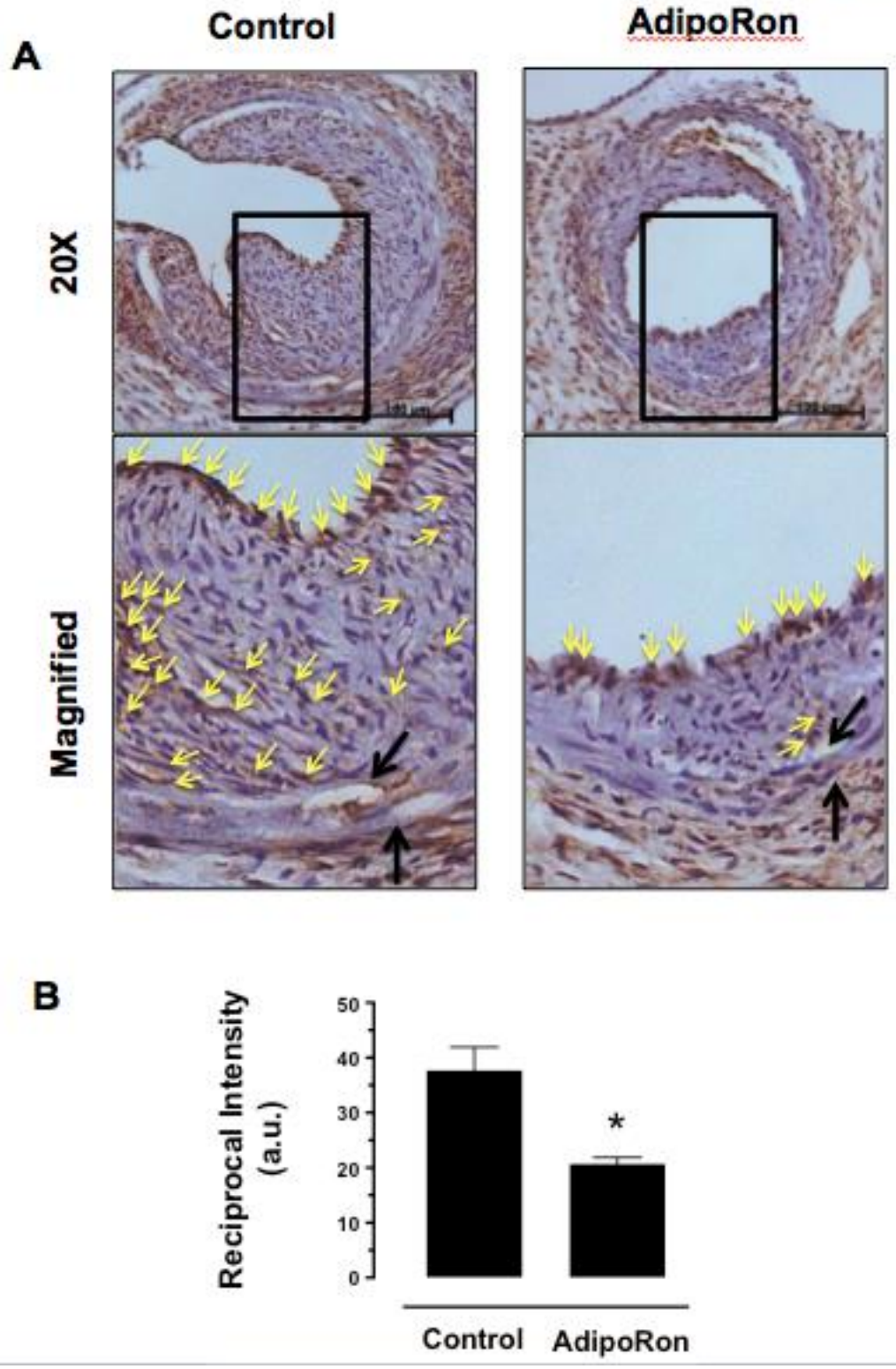
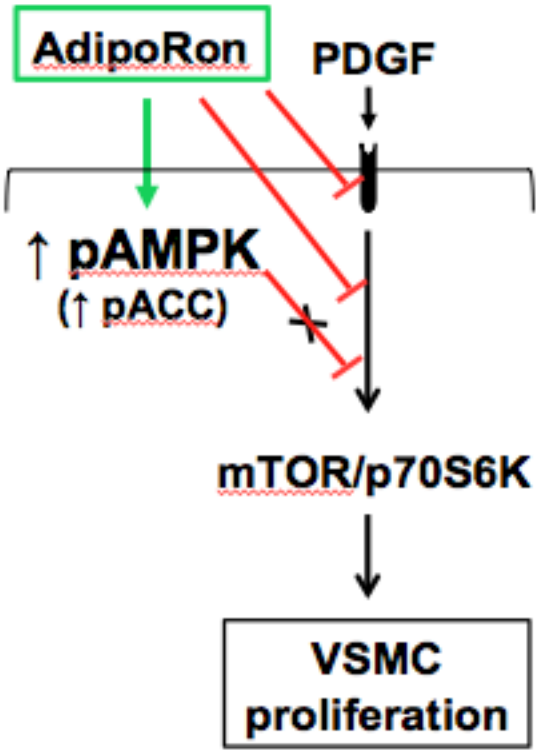

A
Control
AdipoRon
20X
Magnified
B
Reciprocal Intensity (a.u.)
50
40
30
20
10
0
*
Control
AdipoRon

Fig. 2.12. AdipoRon inhibits VSMC proliferation through AMPK-independent inhibition of mTOR/p70S6K signaling. AdipoRon activates AMPK and inhibits basal and PDGF-induced mTOR/p70S6K signaling. AMPK downregulation by target-specific siRNA shows persistent inhibition of mTOR signaling in VSMCs.

Fig. 2.12



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CHAPTER 3

ADIPORON-INDUCED AMPK ACTIVATION DOES NOT MEDIATE ITS VASORELAXANT EFFECT IN ENDOTHELIUM-DENUDED RAT AORTA

Arwa Fairaq, Islam Osman and Lakshman Segar

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ABSTRACT

Adipose tissue-derived adiponectin has been shown to promote endothelium-dependent vasorelaxation through AMP-activated protein kinase (AMPK) and eNOS-mediated nitric oxide production. Recent studies demonstrate that AdipoRon, a small molecule adiponectin receptor agonist, exerts a vasorelaxant effect in arterial smooth muscle. However, the likely regulatory effects of AdipoRon on AMPK activation and the associated relaxant response remain unclear in vascular smooth muscle. **Methods:** Contractility studies were performed using endothelium-denuded rat thoracic aorta. The aortic rings were treated with vehicle (control) or AdipoRon (50 μM) for 15 min. In select experiments, rings were pretreated with an AMPK inhibitor (compound C; 40 mM, 30 min) to determine the intermediary role of AMPK in AdipoRon action. Subsequently, control and treated rings were challenged with cumulative concentrations of PE (10^{-9} - 10^{-4} M) to determine the changes in contractile response. In addition, immunoblot analysis was performed to determine the AMPK phosphorylation state. Our results show that AdipoRon treatment (25, 50 and 100 μM) inhibits PE-induced smooth muscle contraction by 29.4%, 64.4% and 86.1% respectively. Furthermore, compound C, at 40 μM concentration, did not affect AdipoRon-mediated inhibition of PE-induced smooth muscle contractility. AdipoRon significantly decreased Emax and pEC50 of PE by 54.9% and 10.8%. respectively. With compound C pretreatment, AdipoRon significantly decreased Emax and pEC50 of PE by 59.9% and 13.88%, respectively. Immunoblot analysis of the respective aortic rings showed an increase in the phosphorylation of AMPK and its downstream effector, acetylCoA carboxylase (ACC) upon treatment with AdipoRon. The present findings suggest that AdipoRon inhibits vascular smooth muscle contraction through AMPK-independent mechanism. Strategies involving oral administration of AdipoRon may reduce the risk of hypertension in obesity.

Key words:

AdipoRon

Adiponectin

vascular smooth muscle

AMPK

relaxation

Abbreviation:

VSMCs, vascular smooth muscle cells; AMPK, AMP-activated protein kinase; ACC, acetyl CoA carboxylase; Ach, acetylcholine; DMSO, dimethyl sulfoxide; EC, endothelial cell; Emax,; pEC50, negative logarithm of the half maximal effective concentration.

1. Introduction

Hypoadiponectinemia is associated with endothelial dysfunction and an increase in the risk of cardiovascular disease [1] and hypertension [2, 3]. In a clinical setting, low plasma adiponectin level has been reported as a marker for predisposition to hypertension [4]. Importantly, adiponectin knockout mice develop hypertension when maintained on a high-salt diet, and exhibit reduced levels of endothelial NO synthase (eNOS) mRNA in the aorta and low metabolite levels of NO in plasma [5]. At the molecular level, both the full-length and globular adiponectin have been shown to promote endothelium-dependent vasorelaxation *via* increases in NO production through different mechanisms, including eNOS activation[6-9] through phosphorylation of eNOS at Ser 1179 by an AMP-activated protein kinase (AMPK)-dependent mechanism.[10] It also increases eNOS binding to heat shock protein 90 (HSP90) for maximal activity [11]. Both AdipoR1 and AdipoR2 are required for adiponectin-induced NO production [12] and both receptors are expressed in endothelial cells and vascular smooth muscle cells (VSMCs) [13]. Furthermore, adiponectin also enhances eNOS expression [8].

Another alternative to adiponectin administration is suggested to be *via* its receptoractivation. Recently, Okada *et al.* reported for the first time that the orally active adiponectin receptor agonist, AdipoRon, ameliorates type 2 diabetes in diabetic mice through activation of AMPK [14]. To date, the exact role of adipoRon in vasoreactivity remains unclear. AdipoRon has been reported to work *via* different mechanisms when comparing to Adiponectin as suggested by Hong et al [13]. In this study, we hypothesized that adipoRon activates AMPK in vascular smooth muscle but that is not essential for its vasorelaxant effect. In this regard, we aimed to

examine the regulatory effects of AdipoRon on AMPK activation and the associated relaxant response in rat aorta.

2. Material and methods

2.1. Chemicals

Phenylephrine hydrochloride and acetylcholine chloride were purchased from Sigma–Aldrich (St. Louis, MO). Compound C (or dorsomorphin dihydrochloride) was purchased from Tocris Bioscience (Minneapolis, MN). AdipoRon was purchased from Cayman Chemical (Ann Arbor, MI). The primary antibodies for phospho-AMPKα^{Thr172} (2535), phospho-ACC^{Ser79} (11818) and β-actin (8457) were purchased from Cell Signaling Technology (Danvers, MA). HRP-conjugated goat anti-rabbit secondary antibody was purchased from Bio-Rad (Hercules, CA).

2.2. Animals

All animal experiments were performed in accordance with the Charlie Norwood Veterans Affairs Medical Center Institutional Animal Care and Use Committee guidelines and were approved by the committee. Adult male Wistar rats (280–350 g, Charles River Laboratories, Inc., Wilmington, MA) were maintained in a room at a controlled temperature of 23 8C with a 12-h:12-h dark–light cycle. The rats had free access to water and standard rodent chow diet.

2.3. Preparation of aortic rings and isometric tension measurements

As described before [15], after sacrificing the rats, thoracic aorta was isolated and immediately placed in a petri-dish containing ice-cold oxygenated Krebs–Henseleit bicarbonate

(KHB) buffer (118 mM NaCl, 4.7 mM KCl, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 2.5 mM $CaCl_2$, 25 mM $NaHCO_3$, and 11 mM glucose; pH 7.4). The aorta was carefully cleaned free of adherent fat and connective tissue. Endothelium was removed by gently rubbing the luminal surface using a polyethylene tube. Endothelium-denuded aortas were then cut into 2-mm rings. Rings were immediately mounted in organ baths for isometric tension measurements. After passing stainless steel wires through the lumen, aortic rings were suspended from the isometric force displacement transducers (Model FT03; Grass Technologies, West Warwick, RI) and kept immersed in 10 ml of KHB buffer in the organ bath system (eight chambers, Radnoti Glass Technology, Monrovia, CA). The KHB buffer was bubbled continuously with a gas mixture of 95% O_2 and 5% CO_2 and maintained at 378C. Isometric tension was measured as changes in millinewtons (mN) of force using Octal Bridge Amplifier and PowerLab 8/35 data-acquisition system and recorded using LabChart Pro V7 software (ADInstruments, Color- ado Springs, CO). Each aortic ring was gradually stretched to a basal tension of 19.6 mN (2 g) for 1 h and then equilibrated for additional 1.5–2 h. During this equilibration period, KHB buffer was changed every 30 min. After equilibration, the maximal contractile response to 80 mM KCl was determined followed by washes with KHB buffer until the passive basal tension is restored. To verify endothelial denudation in all studies, aortic rings were pre-contracted with phenylephrine (1mM) until the attainment of plateau phase, followed by the addition acetylcholine (1 mM). The absence of relaxant response to acetylcholine confirmed the denudation of rings. The aortic rings were then washed with KHB buffer until the passive basal tension is restored.

2.4. Experimental protocols

In the concentration response experiments, endothelium-denuded aortic rings were pre-treated with PE (10^{-6}M) and then cumulative doses of AdipoRon (5, 25, 50 and 100 μM) or vehicle (control) and the changes in contractile response were determined. In the dose response experiments, endothelium-denuded aortic rings were pre-treated with vehicle (control) or AdipoRon (50 μM) for 15 min. In select experiments, aortic rings were also pre-treated with vehicle (control), compound C (40 μM) for 30 min. Subsequently, control and treated rings were challenged with cumulative concentrations of PE (10^{-10}–10^{-4} M) to determine the changes in contractile response.

2.5. Extraction and quantification of proteins in aortic tissues

After contractility studies, aortic rings were immediately rinsed in ice-cold fresh phosphate-buffered saline, blotted to dryness, snap-frozen in liquid nitrogen, and stored at -80 °C until analysis. Aortic tissues were then thawed and homogenized in 100 ml RIPA lysis buffer containing protease and phosphatase inhibitors (Thermo Scientific, Rockford, IL) using TissueLyser LT (Qiagen, Valencia, CA) at a setting of 50 Hz for 5 min with samples being placed on ice intermittently. The homogenates were incubated at 48C for 1h on a rotator and centrifuged at 1000 g for 10 min at 4 °C to remove tissue debris. The supernatants were mixed with 2X Laemmli sample buffer at a ratio of 1:1 followed by heating at 67.5 °C for 10 min. Proteins were quantified using Bio-Rad DC assay kit (Bio-Rad, Hercules, CA).

2.6. Immunoblot analysis

Aortic tissue samples (20 mg protein each) were electrophoresed using pre-cast 4–12% NuPage mini-gels (Life Technologies, Carlsbad, CA), and the resolved proteins were transferred to nitrocellulose membranes (Hybond C, GE Healthcare Life Sciences, Piscataway, NJ) as described [28]. The membranes were blocked in 5% bovine serum albumin, and probed with the primary antibodies specific for phospho-AMPKα^{Thr172}, phospho-ACC^{Ser79} or β-actin. After extensive washes, the immunoreactivity was detected using specific HRP-conjugated secondary antibodies followed by enhanced chemiluminescence (GE Healthcare Life Sciences). The protein bands were quantified by densitometric analysis using Image J.

2.7. Statistical analysis

Results are expressed as means +/-SEM values. The n value represents the number of animals. To determine E_{max} and EC50 values (pEC50), nonlinear regression analysis was performed using GraphPad Prism software (version 6.01, GraphPad Software, Inc., La Jolla, CA). Statistical analyses of the data among groups with two independent variable were performed by two-way repeated measures ANOVA followed by Bonferroni t test (Concentration dependent and dose response studies). Values of $p < 0.05$ were considered statistically significant.

3. Results

3.1. AdipoRon inhibits PE-induced smooth muscle contractility ex vivo

To examine the effect of AdipoRon on vascular reactivity, endothelium-denuded rat aorta was used. AdipoRon treatment (25, 50 and 100 μM) inhibited PE-induced smooth muscle contraction by 29.4%, 64.4% and 86.1%, respectively **(Figure 3.1).**

3.2. AdipoRon inhibits PE-induced smooth muscle contractility ex vivo through AMPK-independent mechanism

In a recent study, Hong et al. reported that AdipoRon-induced vasorelaxation does not appear to result from AMPK activation [13]. In the current study, we examined the effect of AdipoRon on PE-induced concentration in the presence or absence of compound C (AMPK inhibitor). Results show that AdipoRon significantly inhibited PE-induced concentration in the presence or absence of compound C **(Figure 3.2A).** In addition, AdipoRon significantly decreased Emax and pEC50 values of PE by 54.9% and 10.8%, respectively. With compound C pretreatment, AdipoRon significantly decreased Emax and pEC50 of PE by 59.9% and 13.88%, respectively **(Figure 3.1B-C)**. Thus, AdipoRon-inhibited PE-induced concentration independent of AMPK activation in endothelium-denuded rat aorta.

3.3. AdipoRon activates AMPK in rat aortic smooth muscle

Our previous data demonstrated the ability of AdipoRon to activate AMPK in VSMC in a concentration and time-dependent manner [16]. In this study, we examined the phosphorylation state of AMPK in endothelium-denuded rat aorta. As shown in **Figure 3.3**, western blot analysis

revealed that AdipoRon at 50 μM concentration significantly increased AMPK phosphorylation by 9.4 fold and its downstream effector ACC by 3-fold. In addition, compound C pretreatment nearly abolished AdipoRon induced-AMPK phosphorylation. Together, these studies provide evidence that AMPK phosphorylation is not responsible for AdipoRon inhibition of smooth muscle contractility observed upon PE treatment.

Discussion

The present findings demonstrate that in endothelium-denuded rat aorta, phenylephrine-induced contractile response is markedly attenuated upon treatment with AdipoRon (a small-molecule adiponectin receptor agonist) as a function of concentration. Importantly, AdipoRon treatment led to significant increases in the phosphorylation of AMPK and its downstream target, ACC. While compound C (an AMPK inhibitor) prevented AdipoRon-mediated AMPK/ACC phosphorylation, it did not affect the inhibitory effects of AdipoRon on phenylephrine-induced contractile response. Together, these data reveal that AdipoRon attenuates agonist-induced vascular smooth muscle contraction independent of its role in AMPK activation.

Phenylephrine, an α1-adrenergic agonist, induces vascular smooth muscle contraction through sequential activation of multiple signaling components. In brief, phenylephrine challenge results in the activation of phospholipase C, which catalyzes the conversion of phosphatidylinositol-4,5-bisphosphate to inositol-1,4,5 trisphosphate (IP_3) and diacylglycerol (DAG). IP_3-mediated increase in cytosolic free Ca^{2+} leads to Ca^{2+}-calmodulin complex formation, myosin light chain kinase (MLCK) activation, MLC phosphorylation, and subsequent myosin-actin interaction for cross-bridge cycling [17]. The extent of changes in MLC

phosphorylation is determined not only by MLCK activation, but also by the activation state of RhoA/Rho-associated kinase and the resultant phosphorylation of myosin phosphatase targeting subunit 1 (MYPT1). It is noteworthy that the activation state of MLCK or RhoA/Rho-associated kinase is diminished upon AMPK activation in vascular smooth muscle [18, 19]. Such an event would attenuate agonist-induced MLC phosphorylation and contractile force. Since there is no causal relationship between AMPK activation and the inhibition of contractile response as evidenced in the present study using compound C pretreatment, it is likely that AdipoRon inhibits phenylephrine-induced contraction by targeting MLCK or RhoA/Rho-associated kinase signaling through mechanisms independent of AMPK activation.

A recent study by Hong et al. suggests that AdipoRon exerts direct vasodilator actions on VSMCs via mechanism distinct from adiponectin [13]. Using small arteries from different vascular beds (cremaster muscle arteries, basilar and LAD) from different species, they demonstrate endothelium-independent vasodilatatory effect of AdipoRon via physical removal of endothelium or pharmacological inhibition of known dilator mechanisms. AMPK activation has been reported to mediate the vasorelaxant effect of adiponectin. However, compound C (AMPK inhibitor), did not prevent AdipoRon mediated relaxation. Thus, similar to ourfindings, AdipoRon mediates vasorelaxation independent of AMPK activation.

In our recent study, we reported AMPK activation in human VSMCs upon AdipoRon treatment (5-100 μM). One of the limitations of Hong et al. study was that they did not examine the activation state of AMPK. In the present study, we examined the activation state of AMPK using endothelium-denuded rat aorta. AdipoRon treatment at 50 μM concentration led to an increase in AMPK phosphorylation. Thus, we provide evidence in support to Hong et al's. observations that AMPK phosphorylation is not responsible for AdipoRon inhibition of smooth muscle

contractility. Future studies are needed to investigate the possible direct effect of AdipoRon in VSMC and its potential implications toward antihypertensive effects in an animal model.

Acknowledgements

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Conflict of Interest

The authors declare no conflicts of interest.

Figure legends

Fig. 3.1. Concentration-dependent effects of AdipoRon (5-100 μM) on PE (10^{-6} M)-induced contraction in endothelium-denuded rat aorta. Results are expressed as percentage of PE-induced contraction. Vehicle control experiments (DMSO) show no significant effects on vasorelaxation. The data shown are the means ± SEM. * $p < 0.05$ compared with vehicle control, using two-way ANOVA followed by Bonferroni multiple comparison test (n=4).

Fig. 3.1

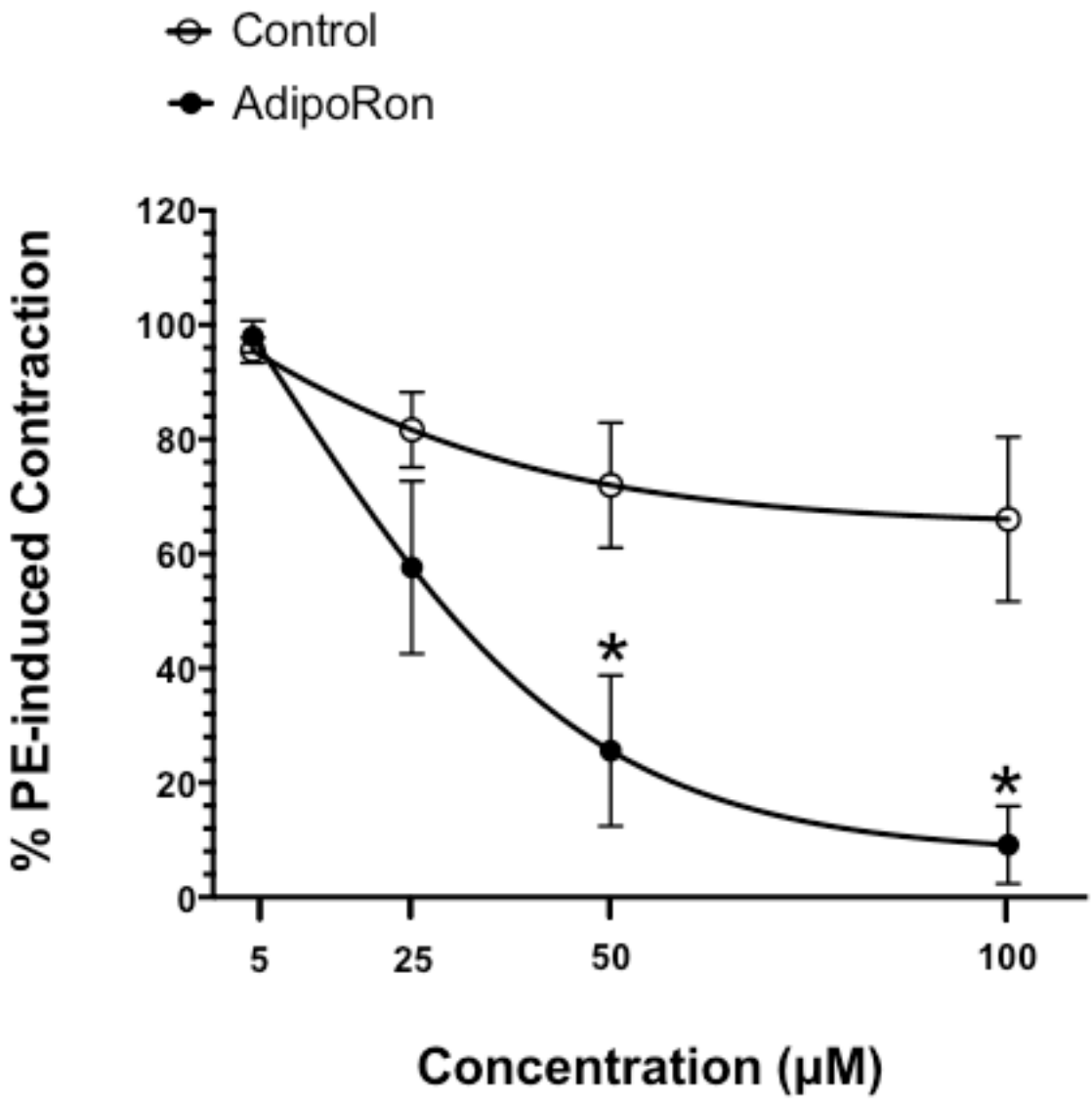

Control
AdipoRon
% PE-induced Contraction
120
100
80
60
40
20
0
5
25
50
100
*
*
Concentration (μM)

Fig. 3.2. Effects of AMPK inhibitor (compound C) on AdipoRon-mediated vasorelaxant response in endothelium-denuded rat aorta. In both A) tracing curve B) Dose-response curve, rings were pretreated with vehicle control or compound C (40 μM) for 30 min followed by exposure to AdipoRon (50 μM) for 15 min. The contractile response to cumulative concentrations of PE (10^{-9}–10^{-4} M) was then determined. C, D) The Emax and pEC50 values for PE-induced contractility were obtained from nonlinear regression analysis. The data shown are the means ± SEM. *, # $p < 0.05$ compared with control or compound C, respectively, using two-way ANOVA followed by Bonferroni multiple comparisons test (n=5-8).

Fig. 3.2A-D

A

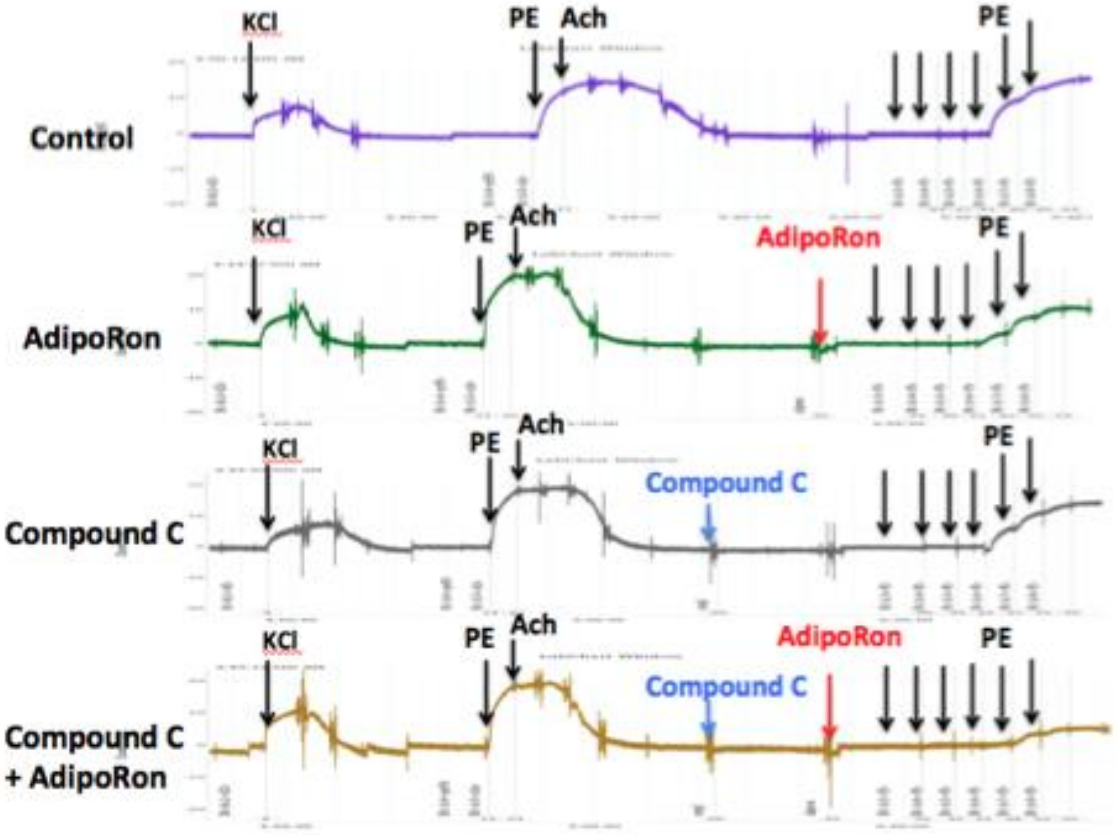

KCl
PE
Ach
PE
Control
KCl
PE
Ach
AdipoRon
PE
AdipoRon
KCl
PE
Ach
Compound C
PE
Compound C
KCl
PE
Ach
AdipoRon
PE
Compound C
+ AdipoRon
Compound C

Fig. 3.2A-D (contd.)

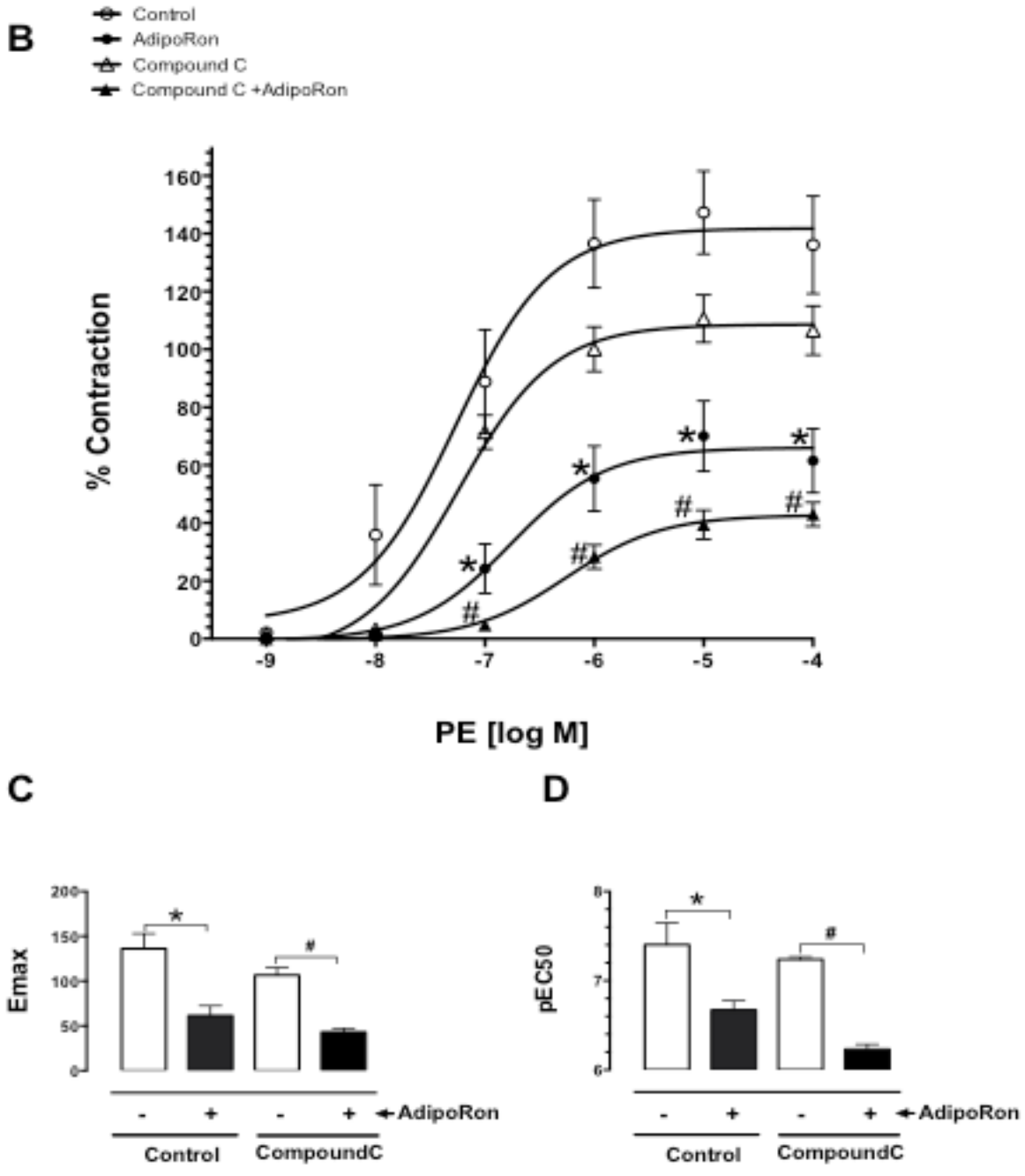
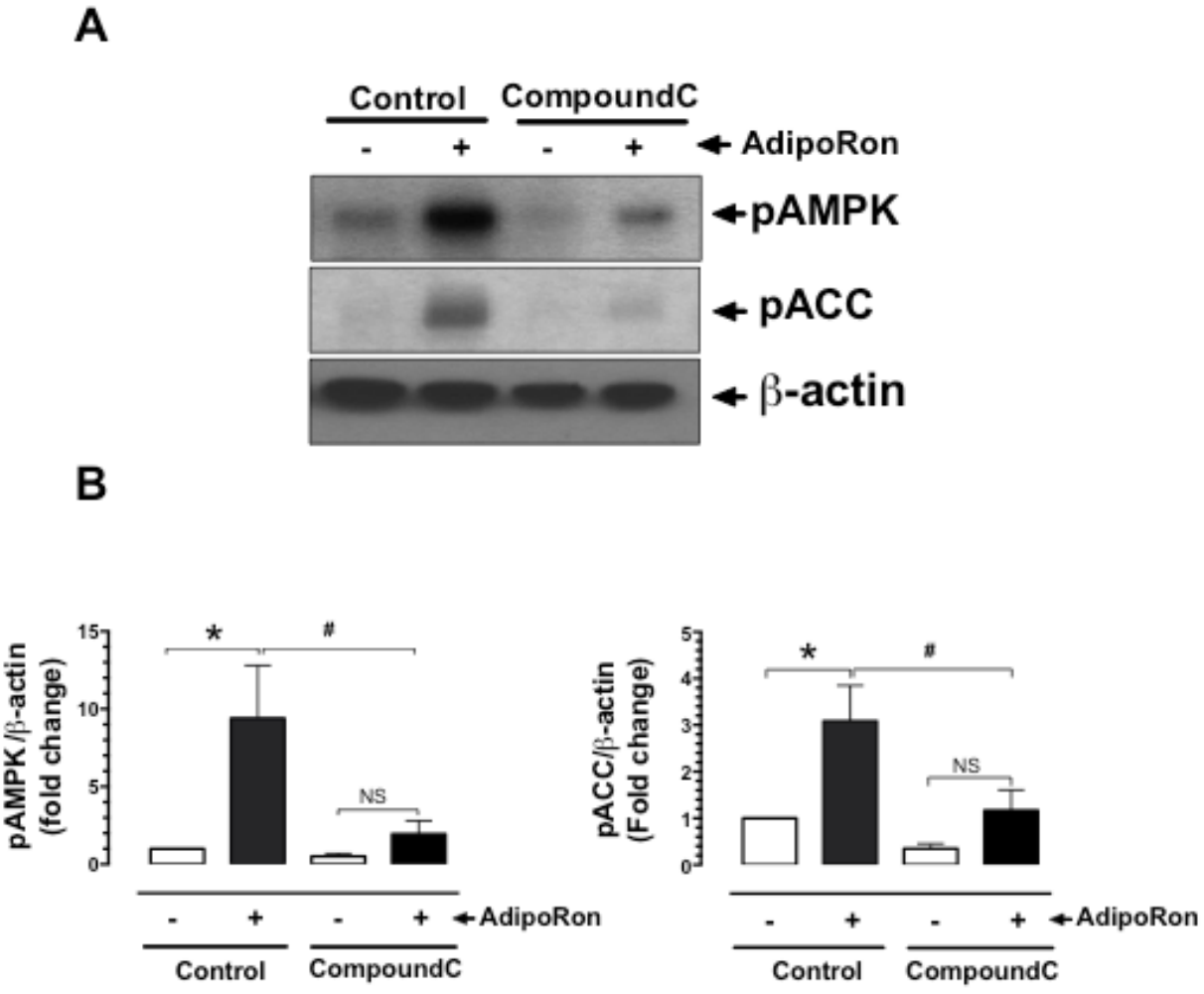


Fig. 3.3. Effects of AdipoRon on AMPK activation in rat aortic smooth muscle: After contractility studies, the respective aortic rings were snap-frozen in liquid nitrogen and subjected to immunoblot analysis using primary antibodies specific for phospho-AMPKα1^{Thr172} and pACC. β-actin was used as an internal control. The data shown in the bar graphs are the means ± SEM. *,# $p < 0.05$ compared with control (Control and AdipoRon), or AdipoRon (AdipoRon and AdipoRon + compound C) respectively, using two-way ANOVA followed by Bonferroni multiple comparisons test (n = 3).

Fig. 3.3A-B


A
Control
CompoundC
- + - +
AdipoRon
pAMPK
pACC
β-actin
B
pAMPK/β-actin (fold change)
15
10
5
0
*
#
NS
- + - +
AdipoRon
Control
CompoundC
pACC/β-actin (Fold change)
5
4
3
2
1
0
*
#
NS
- + - +
AdipoRon
Control
CompoundC

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CHAPTER 4

INTEGRATED DISCUSSION

The aim of this dissertation was to investigate the effect of AdipoRon, a small-molecule Adiponectin receptor agonist, on vascular smooth muscle cell (VSMC) proliferation and contractile response, and to investigate the molecular mechanism towards its therapeutics implications.

In the second chapter, we demonstrated that oral administration of AdipoRon (50 mg/Kg) in C57BL/6J mice significantly diminished arterial injury-induced neointima formation. It was further confirmed with different markers of cell proliferation using immunofluorescence analysis of SM α-actin and Ki-67 immunoreactivity. In addition, we examined the molecular mechanism of AdipoRon in human aortic VSMCs *in vitro*. AdipoRon treatment led to significant inhibition of platelet-derived growth factor (PDGF)-induced VSMC proliferation, DNA synthesis, and cyclin D1 expression. While AdipoRon induced a rapid and sustained activation of AMPK, it also diminished basal and PDGF-induced phosphorylation of mTOR and its downstream targets, including p70S6K/S6 and 4E-BP1. However, siRNA-mediated AMPK downregulation showed persistent inhibition of p70S6K/S6 and 4E-BP1 phosphorylation, indicating AMPK-independent effects for AdipoRon inhibition of mTOR signaling. In addition, AdipoRon treatment resulted in a sustained and transient decrease in PDGF-induced phosphorylation of Akt and ERK, respectively. Furthermore, PDGF receptor-β tyrosine phosphorylation, which controls the phosphorylation state of Akt and ERK, was diminished upon AdipoRon treatment. Together, the

present findings suggest that orally-administered AdipoRon has the potential to limit restenosis after angioplasty by targeting mTOR signaling independent of AMPK activation.

In conjunction with several previous reports that document the ability of adiponectin to inhibit PDGF-induced VSMC proliferation [1-4], the present findings suggest that AdipoRon may provide a novel treatment option to attenuate exaggerated VSMC proliferation. From a mechanistic standpoint, adiponectin has been shown to have multiple targets that contribute to inhibition of VSMC proliferation. First, full length adiponectin binds with PDGF-BB (a potent VSMC mitogen), thereby inhibiting its association with PDGF receptor in VSMCs [3]. In particular, high molecular weight and medium molecular weight forms of adiponectin bind with PDGF-BB, thereby precluding its bioavailability at the pre-receptor level[4]. Second, full length adiponectin has been shown to inhibit autophosphorylation of PDGF receptor-β and the activation of its downstream effector, ERK [3]. Third, adiponectin inhibition of PDGF-induced VSMC proliferation does not require adiponectin receptors (AdipoR1 and/or AdipoR2), as evidenced in studies involving target-specific downregulation of AdipoR1/R2 [4]. Fourth, treatment of VSMCs with adiponectin leads to the activation of AMPK [5, 6], which is implicated in the suppression of VSMC proliferation [7-9]. Although AdipoRon activates AMPK in a robust and sustained manner in VSMCs, it inhibits PDGF-induced VSMC proliferation through an AMPK-independent mechanism.

It is noteworthy that both adiponectin and AdipoRon promote AMPK activation in VSMCs. Yet, they exhibit differences in the temporal activation of AMPK. For instance, recombinant full-length adiponectin has been shown to enhance $AMPK^{Thr172}$ phosphorylation at 30 min and 6-12 hr time points in rat aortic VSMCs [10] and human aortic VSMCs [6], respectively. In a different study, treatment with full-length adiponectin enriched in HMW oligomers, but not

truncated globular adiponectin, leads to a detectable increase in AMPK^{Thr172} phosphorylation at the 24 time point in human coronary artery VSMCs [11]. Regardless of the monomeric or oligomeric forms, adiponectin requires a much longer time frame to enhance AMPK^{Thr172} phosphorylation in VSMCs. In contrast to adiponectin, AdipoRon enhances AMPK^{Thr172} phosphorylation as early as 6 min, which remains elevated in a sustained manner for up to 48 hr in human aortic VSMCs (present study). These findings are consistent with the recent observations of a rapid rise in AMPK^{Thr172} phosphorylation in AdipoRon-treated mouse myoblast C2C12 cell line [12]. As expected, the increase in AMPK^{Thr172} phosphorylation by adiponectin or AdipoRon results in enhanced phosphorylation of ACC (a downstream target of AMPK). It is likely that the differences in the temporal activation of AMPK by adiponectin and AdipoRon are attributable to the differential activation of proximal signaling events, including adipoR1/R2 and LKB1 [13], in VSMCs.

Studies by several investigators, including our recent findings, have shown that AMPK activation results in the suppression of mTOR/p70S6K signaling in different tissues/cell types [14, 15], including VSMCs [7, 8]. Importantly, adiponectin- or AdipoRon-mediated AMPK activation is associated with inhibition of mTOR/p70S6K signaling in VSMCs. Yet, there are differences in the cause and effect relationship between these two signaling events depending on the agonist. For instance, adiponectin-mediated AMPK activation leads to inhibition of mTOR/p70S6K signaling in human coronary or femoral artery VSMCs, as evidenced in experimental approaches involving the use of pharmacological agents (e.g., compound C, an AMPK inhibitor) or target-specific siRNAs [5, 16]. In our study involving AMPK downregulation by target-specific siRNA, AdipoRon-mediated decrease in mTOR signaling remains essentially unaltered. This is revealed by persistent decreases in the phosphorylation

state of p70S6K/S6 and 4E-BP1 signaling components under basal and PDGF-stimulated conditions. Taken together, although AMPK activation by adiponectin is critical for the suppression of mTOR/p70S6K signaling [5, 16], AdipoRon-mediated inhibition of mTOR/p70S6K signaling occurs independent of AMPK activation in VSMCs.

Previous studies have documented the existence of a negative feedback regulatory loop whereby inhibition of mTOR/p70S6K signaling results in the activation of Akt in different cell types under normal and diseased states [17-19]. Notably[5], adiponectin-mediated inhibition of mTOR/p70S6K signaling is associated with Akt activation (at 4-24 hour time points) in human coronary artery VSMCs [11]. In addition, adiponectin overexpression diminishes p70S6K signaling with an accompanying increase in Akt phosphorylation in VSMCs [20]. However, in a different study, adiponectin does not affect Akt phosphorylation in human VSMCs[21]. Furthermore, C1q/TNF-related protein-9 (CTRP9), an adipocytokine and a conserved paralog of adiponectin, fails to activate Akt in human VSMCs [22]. In our study, acute or prolonged treatment with AdipoRon leads to inhibition of PDGF-induced Akt phosphorylation in VSMCs. Together, in the absence of an apparent negative feedback regulatory loop, AdipoRon treatment may inhibit PDGF receptor-mediated proximal signaling components, including Akt in VSMCs.

Although inhibition of mTOR/p70S6K or PI 3-kinase may lead to ERK inhibition [23] or ERK activation [17-19] in different cell types, studies with adiponectin demonstrate its inhibitory effects on PDGF-induced ERK phosphorylation in human VSMCs [3]. In addition, adiponectin attenuates IGF1-induced ERK phosphorylation in rat aortic VSMCs [10]. However, in a different study, adiponectin induces ERK phosphorylation in porcine coronary artery VSMCs [24]. In the present study, AdipoRon treatment between 6 min and 48 hr does not affect the basal phosphorylation of C-Raf and MEK, the kinases upstream of ERK. However, AdipoRon

treatment results in a transient decrease in basal ERK phosphorylation at 6-60 min time points with a reversal to the pre-existing phosphorylation state at 24-48 hr time points. Accordingly, acute exposure to AdipoRon inhibits PDGF-induced ERK phosphorylation, whereas prolonged treatment with AdipoRon does not result in significant changes in PDGF-induced ERK phosphorylation. At this juncture, it is important to note that a transient decrease in ERK phosphorylation followed by its reactivation has been evidenced in recent studies with a PI 3-kinase inhibitor Further studies are clearly warranted to determine AdipoRon regulation of PI 3-kinase activity and its relationship with C-Raf/MEK-independent ERK signaling [25, 26] in VSMCs.

Furthermore, AdipoRon inhibits PDGF receptor-β tyrosine phosphorylation in VSMCs, as has been evidenced in previous studies with Adiponectin [3]. Since adiponectin inhibits PDGF ligand association with PDGF receptor [3, 4] and activates protein tyrosine phosphatase 1B [27], future studies should determine which of these two mechanisms mediates AdipoRon inhibition of PDGF receptor-β tyrosine phosphorylation. Importantly, this decrease in PDGF receptor tyrosine phosphorylation may contribute in part to the observed inhibitory effects of AdipoRon on: i) the association of p85 (adapter subunit of PI 3-kinase) with the activated PDGF receptor; ii) Akt activation; and iii) mTOR/p70S6K/S6 and 4E-BP1 signaling in VSMCs.

In conclusion, our findings strongly suggest that orally-administered AdipoRon has the potential to limit restenosis after angioplasty at the lesion site by targeting VSMC proliferative signaling events, including mTOR/p70S6K. Although AdipoRon activates AMPK reminiscent of adiponectin action, AMPK activation does not play an intermediary role toward AdipoRon-mediated inhibition of VSMC proliferation. In a recent study, AdipoRon has been shown to promote vascular smooth muscle relaxation through AMPK-independent mechanism [28]. In

view of the reported beneficial effects of AdipoRon in improving glycemic control in type 2 diabetic mice [12], strategies that utilize this small-molecule to suppress exaggerated VSMC proliferation may provide a realistic alternative to rapamycin/sirolimus (an mTOR inhibitor), which has been shown to exhibit adverse effects including glucose intolerance and diabetes in rodent models [29, 30].

In the third chapter, we aimed to study the effect of AdipoRon on contractile response, a major player key in cardiovascular disease. As we reported AMPK activation in VSMCs, in this chapter we examined the intermediate role of AMPK activation in AdipoRon-mediated rat aortic relaxation. Using organ bath system, we found that AdipoRon attenuated phenylephrine-induced contractile response in endothelium-denuded rat aorta. AMPK was activated upon AdipoRon treatment but it was not responsible for its vasorelaxant effect.

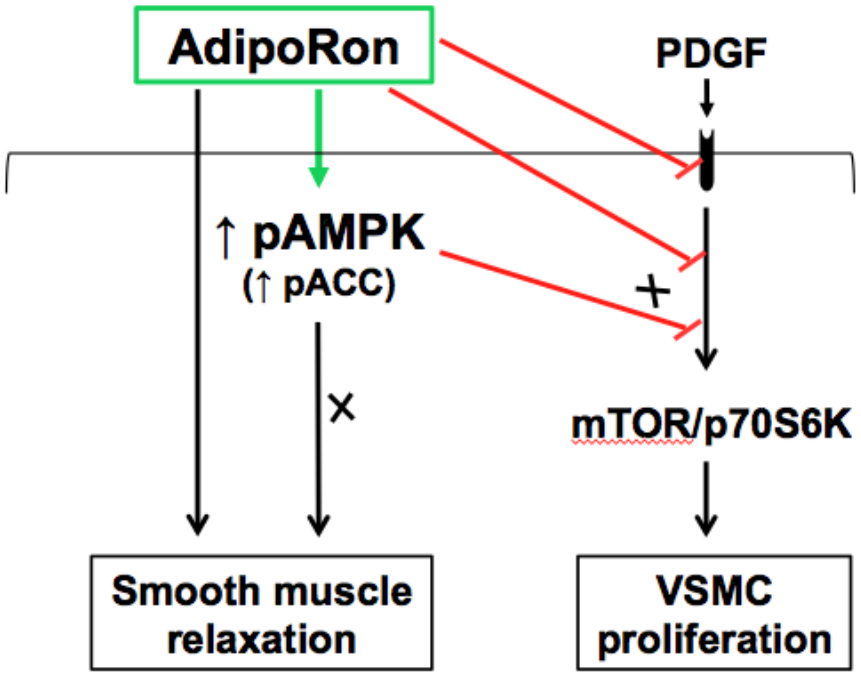
Previous reports suggest that adiponectin induces vasorelaxation through endothelium - dependent mechanisms, including AMPK activation and NO production [31]. However, a recent study by Hong *et al.* suggests that AdipoRon exerts direct vasodilator actions on VSMCs *via* mechanisms distinct from adiponectin [28]. Using small arteries from brain, heart and skeletal muscle and different species, Hong *et al.* showed the endothelium-independent vasorelaxant effect of AdipoRon *via* physical removal of endothelial or pharmacological inhibition of known dilator mechanisms [28]. AMPK activation has been reported to mediate the vasorelaxant effect of adiponectin. However, compound C (AMPK inhibitor), did not prevent AdipoRon-mediated relaxation [28]. Thus, AdipoRon mediates vasorelaxation independent of AMPK activation. Our findings provide additional evidence in support of the observations from Hong *et al.* study, in that AMPK phosphorylation is not responsible for AdipoRon inhibition of smooth muscle

contractility. Strategies involving oral administration of AdipoRon may reduce the risk of hypertension in obesity.

To date, few studies have examined the role of AdipoRon in cardiovascular diseases. Zhang *et al.* showed the benificial role of AdipoRon in attenuating postischemic myocardial apoptosis [32]. Further studies need to be conducted to investigate the effect of adipoRon in other cardiovascular diseases including hypertension. Our findings from femoral artery injury model suggest that oral administration of AdipoRon will provide a noval treatment option to improve restenosis after angioplasty by targeting VSMC proliferative signaling events, including mTOR/p70S6K. Compared to the currently available rapamycin/sirolimus drug-eluting stent formulations, rapamycin treatment has been reported to reduce the recurrence of restenosis [33] but it is associated with impaired re-endothelialization [34] and several adverse effects including glucose intolerance and hyperlipidemia upon chronic administration [29]. However, AdipoRon has been reported to improve insulin resistance and glucose intolerance in type 2 diabetic mice [12], which gives a superior advantage to rapamycin. In addition, it is orally active, so it will provide a realistic alternative to rapamycin. Since reendothelialization is an important component after vascular injury, AdipoRon effects on re-endothelialization should be examined in future studies.

Fig. 4.1. Role of AdipoRon in vascular smooth muscle cell proliferation and smooth muscle contractile response. AdipoRon inhibits VSMC proliferation through AMPK-independent inhibition of mTOR/p70S6K signaling *in vitro.* In addition, AdipoRon-mediated AMPK phosphorylation does not mediate its inhibitory effect on smooth muscle contractility *ex vivo*.

Fig. 4.1



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