Renin-Angiotensin-Aldosterone System and Oxidative Stress in Cardiovascular Insulin Resistance

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Abstract:

Hypertension commonly occurs in conjunction with insulin resistance and other components of the cardiometabolic syndrome. Insulin resistance plays a significant role in the relationship between hypertension, type 2 diabetes mellitus, chronic kidney disease and cardiovascular disease. There is accumulating evidence that insulin resistance occurs in cardiovascular and renal tissue, as well as in classical metabolic tissues (i.e. skeletal muscle, liver and adipose tissue). Activation of the renin-angiotensin-aldosterone system and subsequent elevations in angiotensin II and aldosterone, as seen in the cardiometabolic syndrome contribute to altered insulin/IGF-1 signaling pathways and reactive oxygen species formation to induce endothelial dysfunction and cardiovascular disease. This review will examine currently understood mechanisms underlying the development of resistance to the metabolic actions of insulin in cardiovascular, as well as skeletal muscle tissue.

Introduction

Hypertension is present in about 30% of the adult US population and often occurs in conjunction with insulin resistance and other components of the cardiometabolic syndrome (CMS) (29,115,163,186,190). According to recent data, up to 70 million Americans have insulin resistance, which plays a significant role in the relationship between hypertension, type 2 diabetes mellitus, chronic kidney disease (CKD) and cardiovascular disease (CVD) (69). There is accumulating evidence that insulin resistance occurs in cardiovascular and renal tissue, as well as in classical metabolic tissues (i.e. skeletal muscle, liver and adipose tissue) (125,186,190). This review focuses on currently accepted mechanisms underlying the development of resistance
to the metabolic actions of insulin in CV (Fig. 1 and 3), as well as skeletal muscle tissues (27,190) (Fig. 2).

**Normal actions of Insulin in Cardiovascular (CV) Tissue**

Both insulin and insulin like growth factor (IGF-1) receptors exist in CV tissue (186). Upon binding to specific receptors, they activate a number of downstream signaling systems that result in vasorelaxation (125,188-191) and myocardial glucose uptake and alteration of cardiac energy homeostasis (125,186,190). Activation of the insulin receptor (IR) and the IGF-1 receptor, ligand-activated transmembrane receptors with tyrosine kinase activity, phosphorylates intracellular substrates including insulin receptor substrate (IRS) family members and Shc which, in turn, serve as docking proteins for downstream signaling molecules (27,125). IRS phosphorylation of tyrosine moieties results in engagement of Src Homology 2 (SH2) – domain binding motifs for SH2 - domain signaling molecules including phosphatidyl 3 – kinase (PI3K) and Grb -2. When SH2 domains of the p85 regulatory subunit bind to tyrosine-phosphorylated motifs on IRS-1, this activates the pre-associated p110 catalytic subunit to generate 3,4,5 – trisphosphate (PI(3,4,5) P3. This molecule then binds to the pleckstrin-homology domain in 3-phosphoinositide dependent protein kinase-1 (PDK-1) resulting in its phosphorylation and activation of other downstream serine-threonine kinases including protein kinase B (Akt) and atypical protein kinase C isoforms, which mediate a number of metabolic actions including GLUT-4 translocation to membrane leading to glucose uptake in myocardial tissue and skeletal muscle, as well as NO production in blood vessels (125,186,190,202).

Growth and remodeling responses to insulin and IGF-1 generally involves both the signal transduction and activation of transcription (STAT) and the mitogen activated protein kinase
(MAPK) signaling pathways. This involves tyrosine-phosphorylated IRS-1 or Shc binding to the SH2 domain of Grb-2, which results in activation of the pre-associated guanosine triphosphate (GTP) exchange factor son of sevenless (SOS) and the GTP binding protein Ras, which, phosphorylates/activates extracellular signal-regulated kinase (MEK), and MAPK. Cross-talk from signaling pathways of heterologous receptors, such as the angiotensin II (Ang II) type 1 receptor (AT1R) exert enhancing effects on this growth/remodeling signaling pathways while interfering with the metabolic signaling pathway (186,190,202). Protein tyrosine phosphatases that dephosphorylate the insulin and IGF-1 receptor and IRS-1, as well as lipid phosphatases (i.e. SHIP-2 and PTEN) that dephosphorylate PI (3,4,5) P3 are involved in the negative regulation of insulin and IGF-1 signaling pathways (220). Inappropriate activation of these phosphatases may contribute to insulin/IGF-1 resistance in cardiovascular, as well as liver, skeletal muscle, and adipose tissue (27,202,220).

**Vascular actions of Insulin/IGF-1**

Vascular relaxation effects of insulin/IGF-1 are mediated in part, by endothelial cell production of NO (186,190,224,235,236) (Fig 1A). Insulin/IGF-1 receptor mediation of PI3-K/PDK-1/Akt phosphorylation/activation leads to stimulation of eNOS enzyme activity to produce NO. Phosphorylated/activated Akt, in turn, phosphorylates human eNOS at Ser1177 resulting in enhanced eNOS activity (236). This insulin-mediated activation requires the formation of a ternary eNOS-Heat Shock Protein 90(HSP90)-Akt complex (125,199). Insulin and IGF-1 also increase vascular smooth muscle cell (VSMC) production of NO (13,125,188). Thus, insulin and IGF-1 promote vascular relaxation, in part, via increases in NO bioavailability. Insulin also promotes vascular relaxation by attenuating agonist [i.e. Angiotensin II(Ang II)]-induced increases in cytosolic calcium [Ca^{2+}] and myosin light chain (MLC) kinase activity.
By enhancing MLC phosphatase activity, insulin and IGF-1 reduce MLC kinase activity and thus [Ca^{2+}] sensitive contraction (13,125,174,191,199) (Fig 1B).

**Angiotensin-II Actions on Vasculature**

There is accumulating evidence that Ang II, in addition to its vasoconstriction effects, attenuates the cardiovascular and skeletal muscle metabolic actions of insulin and IGF-1 (115,186,190). The mechanisms involved in these inhibitory effects of Ang II include generation of reactive oxygen species (ROS) and activation of small molecular weight proteins such as RhoA and Rac 1 (9,66,189,190) (Fig. 1, 2). Indeed, there is increasing evidence indicating that Ang II contributes to insulin resistance and other components of the CMS such as hypertension, dyslipidemia, central fat deposition, hepatic steatosis, chronic kidney disease, and proteinuria (69,77,186,190,194,229,230).

Ang II exerts inflammatory effects and promotes vascular growth/remodeling, apoptosis, and fibrosis. There is mounting evidence that increased generation of ROS partially mediate these effects (66,189). These markedly reactive ROS molecules oxidize lipids, protein, DNA, as well as cause cellular injury and enhance vasoconstriction, in part by converting NO to peroxynitrite (ONOO⁻), itself a potent ROS. ROS activate transcription factors such as tumor necrosis factor α (TNF-α), monocyte chemoattractant protein (MCP)-1, interleukin (IL)-6, and C-reactive protein (CRP). TNF-α, in turn, impedes insulin and IGF-1 mediated eNOS activation, as well as the anti-apoptotic actions of insulin and IGF-1(128,186,190).

**Animal Model to Investigate the Role of Ang II in Mediating Insulin/IGF-1 Resistance:**

Our laboratory has utilized the transgenic TG(mRen2)27 rat, that harbors the mouse renin gene and displays activated tissue renin-angiotensin-aldosterone (RAAS) with increased Ang II levels, and increased plasma mineralcorticoids, to evaluate the role of increased tissue Ang II and
mineralcorticoids in mediating CVD, as well as, skeletal muscle insulin resistance (Fig 3) (18,229, 231). Indeed, this rodent model develops proteinuria (77, 231), as well as insulin resistance (18), fatty liver steatosis, and hypertension (18,77,229,231) making it a relevant model of the CMS.

Recent studies in our laboratory have observed that vasculature from young Ren2 rats exhibits increased NADPH oxidase activity, ROS levels (Fig 4), lipid peroxidation, inflammation (increased expression of TNF-α and CRP), and indices of apoptosis compared with Sprague Dawley rats (230). Further, in the vasculature there is marked reduction in insulin stimulation of Akt signaling eNOS Ser1177 phosphorylation/activation. These abnormalities are markedly improved by in vivo treatment with an AT1R blocker or the superoxide dismutase (SOD)/catalase minetic tempol. Available data suggest that vascular RAAS activation and insulin/IGF-1 resistance perpetuate each other and concordantly contribute to endothelial dysfunction, vascular inflammation/remodeling, and hypertension (230). Similar observations have also been made in the left ventricle of hearts taken from young insulin resistant Ren2 rats (33,192,231).

Insulin and Ang II in the Heart

Insulin regulates metabolism in cardiovascular tissue by modulating glucose uptake and utilization, glycogen synthesis, lipid metabolism, proliferation, contractility, remodeling and apoptosis in cardiomyocytes (Fig 2). Insulin and IGF-1 exert a number of metabolic and functional effects on the heart (2,22,33,62,64,70,84,98,105,117,137,146,153,163-165,179,180,192,198,222)(Fig. 2). Both peptides regulate glucose uptake, glycogen and protein synthesis, growth and lipid metabolism (2,98,137). As in skeletal muscle, glucose uptake in cardiomyocytes involves mobilization of the insulin responsive glucose transporter GLUT4 via a PI3K-Akt signaling pathway (2, 98) (Fig 2). Further, in cardiomyocytes, insulin stimulation of
the PI3K-Akt pathway results in phosphorylation and nuclear exclusion of the forkhead transcription factor FOXO-1, which further modulates glucose and lipid metabolism (125, 137).

Insulin and IGF-1 normally enhance cardiac contractility (22, 153, 163-165, 186, 190) via signaling through the PI3K-Akt pathway. This signaling is associated with enhanced ionic calcium (Ca$^{2+}$) influx via activation of L-type Ca$^{2+}$ channels and reverse mode Na$^+$/Ca$^{2+}$ exchange (117, 222). Insulin and IGF-1 also enhance cardiomyocyte myofilament Ca$^{2+}$ sensitivity (42). Increases in myocardial NO production through the PI3K-Akt-eNOS pathway also appears to contribute to the inotropic effects of these peptides (62, 164) (Fig 2).

Insulin and IGF-1 induced increases in myocardial contractility result in increased oxygen consumption (198). Cardiac oxygen demand is a potent determinant of myocardial blood flow (MBF), and insulin and IGF-1 enhance MBF and promotes capillary recruitment in the heart (84, 198). Hyperinsulinemia increases MBF, particularly in areas of the myocardium associated with high rates of glucose uptake (64, 105, 146, 179, 180) (Fig. 4). These observations suggest coupling between the metabolic and coronary vascular actions of insulin and IGF-1 in the heart, with increases in capillary recruitment and MBF enhancing insulin-stimulated increases in delivery of insulin and glucose. These actions of insulin and IGF-1, as well as their direct effects on cardiomyocytes, also enhances glucose transport (2, 98, 137).

Insulin and IGF-1 also regulate developmental and physiological growth and remodeling of the heart (41, 78, 94, 102, 142, 163, 223, 234) (Fig. 2). The peptides accomplish these effects by signaling through the PI3-K-Akt pathway (78, 102, 223). Downstream from Akt, activation of mammalian target of rapamycin (mTOR) promotes cardiac growth, while suppression of GSK3-β, as well as FOXO phosphorylation also modulates cardiomyocyte growth (78, 223). Signaling through the Akt pathway also exerts anti-apoptotic effects on the myocardium (163), by
negatively regulating apoptotic factors, and positively regulating factors that induce survival genes.

Indeed, one apoptotic signaling system modulated by the Akt pathway involves phosphorylation and nuclear exclusion of the FOXO subgroup of the forkhead family of transcription factors. Insulin and IGF-1 also promote survival by direct phosphorylation / inactivation of BAD, a member of the Bcl-2 family, which promotes apoptosis by binding to and antagonizing the action of prosurvival members of the family such as Bcl-2 and Bcl-XL. Insulin/IGF-1 activation of Akt may also interfere with stress-activated protein kinases such as JNK, p38, and MAP kinase pathways critically involved in the induction of apoptosis following exposure of cardiomyocytes to physical stress stimuli (94,234). Finally, Akt activation increases expression of c-FLIP, a caspase-8 homologene that inhibits TNF receptor family-induced apoptosis (142). In conditions of insulin resistance/ hyperinsulinemia, pathological cardiomyocyte hypertrophy is promoted by interactions of insulin/IGF-1 with growth factors such as Ang II, catecholamines, endothelin and mineralocorticoids to stimulate signaling pathways involving MAPK, p38, MAP kinase JAK/STAT, and small molecular weight G proteins: Rho and Ras (163,180,186,188,190).

**Insulin Resistance and Cardiovascular Disease: Role of RAAS and Other Factors.**

There is accumulating evidence that hypertension predisposes an individual to diabetes independently of other CVD risk factors, such as obesity (186,187,190). Clinical evidence supports a link between insulin resistance/ hyperinsulinemia and hypertension, including positive associations between blood pressure and fasting insulin levels in patients with essential hypertension (42,118,162,186,190). Mechanisms to explain this linkage include cellular abnormalities in insulin signaling (186,190), cellular cation alterations, enhanced sympathetic
nervous system activity (162), enhanced RAAS activity (186,190), as well as inflammation and oxidative stress (186,190). Importantly, resistance to the metabolic and proliferative actions of insulin appears to be differential. Indeed, a seminal feature of insulin resistance is impairment in PI3K/Akt signaling metabolic pathways, while other insulin signaling growth pathways including RAS/MAPK/JAK/STAT signaling are not inhibited (36,89,150,186,190). In the vasculature, this leads to diminished endothelial mediated vasodilatation and increased growth remodeling and atherosclerosis (18,89,50).

In addition, pro-inflammatory effects of chronically elevated levels of glucose and fatty acids (FA) contribute to endothelial dysfunction, chronic low-grade inflammation and insulin resistance. For example, exposure of the vasculature and myocardium to elevated levels of free fatty acids leads to impaired insulin signaling (47,226), enhancement of vascular RAAS (227) and oxidative stress (83), as well as impaired insulin-stimulated eNOS activity and NO production (47). Chronic hyperglycemia also increases oxidative stress in the vasculature (166). Increased ROS induced by hyperglycemia and dyslipidemia further impairs insulin signaling, decreases NO bioavailability, reduces cellular tetrohydrobiopterin levels and promotes generation of superoxide by eNOS.

Role of RAAS in Vascular Insulin Resistance.

As noted previously, physiologic concentrations of insulin increase vasodilatation through NO release and exert anti-oxidant and anti-inflammatory effects via signaling through the PI3-K/Akt metabolic pathway(186,190). Ang II and mineralcorticoids, in contrast, cause vasoconstriction and enhance the expression of pro-inflammatory cytokines, adhesion molecules, growth and inflammatory pathways (80,85,86,135,153,192,207,208). Further, Ang II and
aldosterone interfere with many of the metabolic signaling actions of insulin and IGF-1 in the cardiovascular system (9,80,85,86,102,135,153,186,188-190,207,208,230).

Ang II, acting through the AT<sub>1</sub>R, increases generation of ROS in the vasculature, primarily through activation of membrane-bound NADPH oxidase enzyme complex (Fig 1A and B) (8,16,31,57,63,101,112,141,155,159,189,201,206,213). Infusion of Ang II impairs endothelium-dependent vasorelaxation (31), and this impairment is corrected by co-administration of superoxide dismutase (SOD) (105), indicating the critical role of ROS in Ang II mediated endothelial dysfunction (189). Ang II stimulated ROS inhibits insulin/IGF-1 signaling through the PI3-K/Akt signaling pathway to activate eNOS (13,125,199,224,235,236). Further ROS generated by Ang II inactivate NO (20,120,152,203), and the resultant decrease in bioavailable NO, in turn, up-regulates the AT<sub>1</sub>R on vascular cells (81). This creates a cycle of impaired endothelium-derived vasodilation and increased Ang II mediated vasoconstriction. Ang II also stimulates Rho A/Rho-kinase activation, which decreases eNOS expression, in part, by decreasing eNOS messenger RNA stability (122,200) (Fig 1A). Ang II, acting via its AT<sub>1</sub>R, increases VSMC contraction by increasing intracellular Ca<sup>2+</sup> [Ca<sup>2+</sup>] and Ca<sup>2+</sup> -MLC sensitization (129,233) (Fig 1B). Both processes are mediated, in part, by Ang II stimulated generation of ROS in EC and VSMC (189,205,233). Ang II also increases Ca<sup>2+</sup> MLC sensitization by stimulating Rho kinase (ROK) activity in VSMC, whereas insulin and IGF-1 induces relaxation by increasing EC production of NO, and by reducing Ca<sup>2+</sup> MLC sensitization (175). Ang II decreases the ability of insulin and IGF-1 to decrease Ca<sup>2+</sup> MLC sensitization by activating Rho kinase, which phosphorylates the myosin binding protein (MBP) and thereby inhibits the ability of these peptides to dephosphorylate Ca<sup>2+</sup> MLC, which leads to increased Ca<sup>2+</sup> MLC phosphorylation (186,190) (Fig 1B). This concept is bourne out by the observation
that increases in ROK and a decrease in MBP activity occurs in Ang II mediated (30) and insulin resistant (176) hypertensive rodents.

Increased ROS also activates multiple redox signaling pathways including NF-κB (127). NF-κB, in turn enhances other Ang II mediated inflammatory responses by up regulating other inflammatory molecules such as TNF-α, monocyte chemoattractive protein (MCP-1), and C-reactive protein (CRP) (72,124). TNF-α activates several serine kinases including JNK, IκKβ and IL-1β receptor-associated kinase (91), which directly or indirectly increases serine phosphorylation of IRS-1/2, leading to decreased PI3K/Akt signaling responses, and subsequent impaired insulin/IGF-1 stimulation of eNOS, production of NO, and vasodilatation (6,51,95,96). TNF-α increases expression of other inflammatory substance including IL-6 and CRP. CRP, in turn, appears to attenuate insulin-stimulated NO production in EC by increasing phosphorylation of IRS-1 at the Ser307, indirectly by enhancing ROK and JNK signaling (6,217). CRP also upregulates VSMC AT1R (225) and increases the expression of VCAM, ICAM, E-selectine and MCP-1 in EC (144), thus counterbalancing the anti-atherosclerotic and vasodilatory effects of insulin/IGF-1 stimulated NO production.

In addition to stimulating membrane NADPH oxidase in vascular cells, Ang II in conjunction with other cellular stresses, may increase ER stress (139) and mitochondrial oxidative stress (186). In addition to the impact of these inflammatory changes with regard to vasomotion and atherosclerosis, alterations in microvascular blood flow may impact cardiac, adipose tissue, skeletal muscle and liver blood flow. In adipose tissue and liver, microvascular inflammation may affect be contributing to adipose tissue inflammation (increased macrophages) (35) and nonalcoholic fatty liver disease (1), conditions frequently associated with alterations in the RAAS system and insulin resistance (183). Another maladaptive effect of increased
oxidative stress is enhanced DNA strand breaks (88) and depletion of cellular nicotinamide -
adine dinucleotide (NAD⁺) (88,140). Reductions in NAD⁺ concentrations further result in
depletion of cellular ATP levels and, hence, cellular energy levels (88,140). Increased oxidative
stress can also be accentuated by CuZn superoxide dismutase (SOD) deficiency in response to
Ang II (43,45). Indeed, deficiency in CuZn SOD, the most abundant of 3 SOD isoforms, is
associated with increases in ROS and vascular dysfunction (44).

Effect of RAAS on cardiac insulin signaling, structure and function

As previously noted, insulin and IGF-1 generally exert beneficial effects on myocardial
mechanical-electrical coupling and both diastolic and systolic function
(22,62,64,84,105,117,146,153,163-165,179,180,198,222). These beneficial effects appear to be
lessened in conditions of RAAS activation in the heart (33,179,192,231). Many of these
beneficial effects of insulin and IGF-1 are mediated largely by PI3K/Akt signaling
(41,78,94,102,142,188,223,234) and Ang II opposes insulin/IGF-1 mediated signaling through
this pathway (18,33,77,184,192,194,229-231) (Fig 2).

There are several mechanisms whereby cardiac RAAS activation inhibits the beneficial
metabolic effects of insulin and IGF-1. Ang II plays a seminal role in the genesis of cardiac
hypertrophy, interstitial fibrosis and left ventricular dysfunction
(34,46,65,146,172,173,178,204,216,219) (Fig 2). Ang II receptors have been characterized in
cardiomyocytes and cardiac fibroblasts (34,146,172,173,216,219), as well as the endothelial
lining of the coronary arteries (65,146,178,216,237). Although both AT₁ and AT₂ receptors are
present on cardiac and coronary vessel tissue, most of the adverse effects of Ang II on
hypertrophy, fibrosis and LV dysfunction are mediated through the AT₁R (146,237). There are
increasing experimental data suggesting that many of the detrimental effects of both Ang II and
aldosterone are triggered by redox cycling of ROS, generated by a membrane NADPH oxidase dependent pathway, as well as mitochondrial generated ROS (11,24,25,58,33,37,38,75,123,126,146,153,156,189,192,231). In cardiomyocytes, Ang II stimulates a phagocytic type NADPH oxidase, which is comprised of a membrane-bound p22phox heterodimer and 4 regulatory subunits: p40phox, p47phox, p67phox, Nox 2, and the small molecular weight G protein Rac 1 (37,130,136,138). Ang II activation of the NADPH oxidase enzyme affects cell signaling responses and facilitates cardiac remodeling and hypertrophy (11,24,38,153) as evidenced by attenuation of these pathological effects following treatment with free radical scavengers (153,195,231,237) or AT1R blockade (146,173,219,231,237).

In a recent investigation, it was hypothesized that chronic Ang II overexpression in the heart was associated with structural and functional abnormalities that are driven by NADPH oxidase mediated generation of ROS (231). This notion was evaluated by in vivo treatment with either an AT1R blocker or a superoxide dismutase (SOD)/catalase mimetic of a rodent model of chronically elevated tissue levels of Ang II, the transgenic TG(mRen2)27 rat (Ren2). Results of this investigation indicated that the hypertensive, insulin resistant Ren2 rat manifests increased oxidative stress in concert with structural and functional changes in the heart. Membrane NADPH oxidase activity and immunostaining of NADPH oxidase subunits p22phox, NOX 2, and Rac1 were significantly increased in the Ren2, in conjunction with increased levels of myocardial tissue oxidative stress. Structurally, septal wall thickness measured by in vivo cine magnetic resonance imaging was significantly increased. Additionally, light microscopy revealed substantial left ventricular coronary artery perivascular fibrosis. Citrate synthase activity and transmission electron microscopy demonstrated significant increases in mitochondrial number in Ren2 left ventricle tissue. Systolic function was also diminished in the
Ren2 compared to the Sprague-Dawley control. These effects were abrogated by both the AT$_1$R blockade and the SOD/catalase mimetic; highlighting the role of Ang II in the activation of NADPH oxidase and importance of ROS on cardiac remodeling and dysfunction. While the observations are novel in the Ren2 model, previous studies have shown that Ang II increases ROS in cultured myocardial fibroblasts and cardiomyocytes (7,109,113).

The limited endogenous antioxidant capacity, both enzymatic and non-enzymatic, of myocardial tissue renders it highly susceptible to oxidative stress induced injury (50). Thus, increased oxidative stress in the heart has been causally linked to ventricular hypertrophy, diastolic and systolic functional abnormalities, as well as abnormal metabolic signaling (50,136). Indeed, insulin stimulated Akt / protein kinase B phosphorylation / activation is significantly suppressed in the Ren2 myocardial tissue and inversely correlated to Rac1 expression and NADPH oxidase activity (231). In the heart, Akt activation is critical for proper regulation of proteins responsible for growth, metabolism, survival, and cardiac function (5,32,41,182). Akt activity in the heart is regulated by nutritional status, insulin, pressure overload, and redox status (5,32,41,182). Optimal Akt signaling, while important for physiological growth, impedes pathological cardiac hypertrophy (5,32,41,182). Restored Akt activation / phosphorylation, along with abrogation of cardiac hypertrophy and dysfunction, were observed following reductions in tissue oxidative stress by treatment with either the AT$_1$R blockade or SOD / catalase mimetic.

Investigators have evaluated the efficacy of direct renin inhibition on cardiac oxidative stress and remodeling in the Ren2 model of chronic Ang II overexpression using the novel non-peptide renin inhibitor aliskiren (33). The specificity of aliskiren prevents its use in conventional rat models; however, the Ren2 overexpresses murine renin, which is recognized by aliskiren
Renin is the rate limiting step in the generation of Ang II (148,158,232), thus renin inhibition should reduce tissue Ang II levels, as well as, abrogate any direct renin effects. Previous studies have demonstrated the cardioprotective properties of AT₁R blockade (146,231). However, AT₁R blockade generates a reactive release of renin due to decreased inhibition of renal juxtaglomerular cells, which may promote myocardial injury (158,232). Thus, reduction of Ang II levels via direct renin inhibition is of potential therapeutic importance as it blocks the RAAS at its source (148,158,232).

Myocardial tissue from untreated heterozygous male Ren2 transgenic rats display significantly increased levels of ROS generated by increased NADPH oxidase activity as evidenced by increased immunostaining for the NADPH subunits p47phox and Rac1, as well as 3-nitrotyrosine. Translocation of the small GTP binding protein Rac1 and p47phox to the cell membrane is necessary for assembly and activation of NADPH oxidase, which has been directly implicated in Ang II induced cardiac hypertrophy (3,19,50). 3-nitrotyrosine results from ROS scavenging of nitric oxide produces peroxynitrite (ONOO⁻), which binds to protein tyrosine moities to produce stable 3-nitrotyrosine, a surrogate marker of oxidative stress (33,70,77,192,230,231).

Direct renin inhibition in Ren2 animals significantly reduced levels of myocardial oxidative stress as evidenced by decreased immunostaining for Rac1, NADPH subunit p47, as well as 3-nitrotyrosine. Thus, renin blockade effectively attenuated myocardial oxidative stress likely by down-regulating NADPH oxidase. Additionally, interstitial and perivascular fibrosis were evaluated by Verhoeff-van Gieson (VVG) staining, which is specific for elastin, collagen, connective tissue, and nuclei. As previously described, the Ren2 exhibited increases in myocardial interstitial and perivascular fibrosis, which were abrogated by renin inhibition (33).
The results of this study complement previous studies, evaluating the effects of renin inhibition. In these studies, renin inhibition has been shown to lower blood pressure in spontaneously hypertensive rats, double transgenic rats (dTGR), marmosets, and hypertensive humans (135,148,149,218). Renin inhibition has also been shown to significantly improve cardiac hypertrophy, diastolic and systolic dysfunction, as well as reduce albuminuria and kidney inflammation/damage in the double transgenic rat model (148). However, the beneficial effects of in vivo aliskiren administration on measures of myocardial oxidative stress, cellular remodeling, and fibrosis in the Ren2 model of tissue RAAS overactivation provide additional evidence for a critical role for the RAAS system in cardiac remodeling and hypertrophy.

**Myocardial Metabolic Signaling**

Cardiac tissue is capable of remarkable metabolic flexibility. In the normal heart, approximately 10%–40% of the adenosine triphosphate (ATP) is produced via tricarboxylic acid cycle glycolysis, whereas the remaining 60%–90% is derived from β-oxidation of fatty acids. However, energy substrate preference is dynamic to fulfill the energetic requirements of one of the most metabolically active organs in the body. Substrate utilization in the myocardium is dependent upon vascular perfusion, energy demand, substrate availability, and local/systemic hormonal changes (128,215). For example, the heart preferentially shifts toward glucose rather than fatty acid or lactate metabolism under ischemic conditions (128). Similarly, recent studies have shown that the hypertrophied heart is characterized by a marked shift in substrate preference from typical fatty acid to primarily glucose metabolism (214), which is more readily converted to ATP. However, in conditions of insulin resistance, glucose metabolism is impaired and the heart is forced to revert to fatty acid and ketone catabolism (146) resulting in structural and other biochemical changes that ultimately lead to left ventricular hypertrophy and diastolic
(impaired relaxation) and systolic dysfunction (10,79,111,137,147,160,212). In fact, human studies have demonstrated that short term depletion of serum free fatty acids (FFA) in failing hearts results in impaired cardiac work as the heart typically responds to decreases in FFA by increasing glucose metabolism (209).

*In vivo* evaluation of myocardial substrate preference using Positron Emission Tomography (PET) is an area of emerging interest. Previously, PET was primarily used to evaluate myocardial viability by the preservation of glucose metabolism following infarction, ischemia, or injury using 18F-deoxyglucose (18FDG) (67,68). However, advances in PET imaging technology now allow clinicians and investigators to evaluate myocardial metabolic flexibility by measuring myocardial fatty acid uptake (MFAUp), utilization (MFAU), and oxidation (MFAO) using radiolabeled 1-11C-palmitate (14,15,23,40,92,107,146,185) and myocardial efficiency using 11C acetate (12). Numerous studies have evaluated myocardial insulin sensitivity in humans (48,71). The application of nuclear medicine techniques to laboratory animals is another area of emerging interest. For example, our laboratory uses this methodology to measure insulin stimulated myocardial glucose uptake with 18F-deoxyglucose (18FDG) using the Micro-PET™ rodent imaging system and small animal MRI (64) (Fig 5).

**Mineralocorticoids, Cardiovascular Disease, and Insulin Action**

Aldosterone exerts a number of maladaptive effects on the vasculature, heart, and traditional insulin sensitive tissues, such as skeletal muscle (4,17,21,26,28,39,49,52-56,59-61,73,76,82,87,90,93,97,99,100,102,108,110,114,116,121,132-134,142,155,157,168-171,177,181,194,196,197,221,228,238) (Fig 1-3). These maladaptive effects on the vasculature are mediated by both genomic and nongenomic actions of this hormone (53,119,194). Effects on the vasculature include enhancement of tyrosine phosphorylation inositol phosphate activation,
increased Na+/H+ exchange, and alkalinization of VSMC (4,49,76,114,228). Indeed
mineralcorticoid receptors (MR) have been identified in the vasculature (61). As in other tissues,
many of the adverse effects of mineralcorticoids on the vasculature appear result from increased
oxidative stress (177).

Aldosterone infusion into rats results in impaired endothelium-dependent relaxation and
this is associated with increased oxidative stress in the vessel (155). Chronic treatment with
aldosterone caused impaired endothelium dependent vasodilation in these rats (17). Further,
treatment with the MR blocker spironolactone (SP) has been shown to improve endothelium
dependent vasorelaxation in rodents (221) and humans (54). It has been reported that
aldosterone induces endothelial cell swelling, with concomitant increases in protein leakage
through intracellular gaps that may result from increased apical membrane tension (133,134) and
that these processes are blocked with SP (132). There is accumulating evidence that Ang II and
mineralcorticoids have interactive effects on the vasculature (Fig 1A and 1B). Mineralcorticoids
upregulate Ang II receptors in VSMC (211) and signalling of Ang II is amplified by exposure to
mineralcorticoids (210,211). Both Ang II and aldosterone stimulate vascular growth and
remodeling (82,116,121) perhaps mediated through MAP kinase and ROS signalling
(116,121,155). Further, blockade of both MR and AT1R protect against generation of excess
ROS and resultant vascular remodeling (26). Other studies demonstrate that aldosterone may
interfere with insulin signaling in various tissues (26,99), although the effects of
mineralocorticoids alone and in conjunction with Ang II on insulin signaling in vascular tissue
remain to be elucidated.

Mineralcorticoids in the Heart
There is considerable evidence that mineralcorticoids contribute to abnormal cardiac remodelling, including fibrosis and perivascular inflammation (21,39,55,56,59,60,73,90,93,110,157,167-171,181,196,197,238). Both cardiomyocytes and fibroblasts express MR with high affinity for both corticosterone and aldosterone (110,181,157). The inflammatory effects of corticosterone and aldosterone in the heart are partly mediated by an interaction with the renin-angiotensin-aldosterone system (RAAS), as well as effects directly mediated through MR activation (39,55,56,73,90,167,168,170,196,238). For example, in rats treated with aldosterone and high dietary salt, the AT1R expression and ventricular density of the AT1R have been observed to increase (90). Additionally, mineralcorticoids increase expression of angiotensin converting enzyme (ACE) in cardiomyocytes from adult rat primary (196) and in cultured rat fetal cardiomyocytes (73). Recent data from several laboratories suggest that MR activation may potentiate the pro-inflammatory/fibrotic effects of AT1R signaling by enhancing the cardiac oxidative stress induced by Ang II (90,93,196,238). Further, animal studies have shown that MR antagonism reduces oxidative stress, inflammation, and fibrosis independent of blood pressure effects (55,56,168). These beneficial effects of MR blockade are mediated, in part, through inhibition of NADPH oxidase activity (97,100,142) (Fig 2).

Mineralcorticoids and Insulin Sensitivity

There is accumulating data from human and animal studies that excess mineralcorticoids impair insulin signaling in a number of tissues. For example, aldosterone excess in patients with primary aldosteronism is related to impaired glucose homeostasis (52), as well as insulin resistance (28). Several recent publications (99,194), as well as recent data from our laboratory (102) suggest that these detrimental effects on insulin signaling are mediated by
inflammatory/oxidative stress effects of mineralcorticoids. Indeed, in the TG(mRen2)22 rat, which manifests insulin resistance (18), in vivo MR antagonism with subpressor doses of SP substantially improve ex-vivo insulin stimulated increases in glucose uptake in skeletal muscle, a phenomenon that is linked to reductions in NADPH oxidase activity and attenuation of ROS in soleus muscle tissue (102). Future work will focus on the impact of MR and glucocorticoid receptor antagonism and its impact on insulin and IGF-1 signaling in cardiovascular tissue.

**Conclusions**

In summary, activation of the RAAS contributes to altered insulin/IGF-1 signaling pathways that lead to reactive oxygen species formation, endothelial dysfunction and pathological growth and remodeling. Both AT1R and MR activation contribute to downstream signaling pathways that attenuate insulin signaling mechanisms in the heart, vasculature, and skeletal muscle that collectively alter physiologic regulation of transcriptional and translational maintenance of cell metabolism. Collectively, these changes contribute to CVD as seen in the CMS.

**Figures Legends:**

**Figure 1 A)** Vascular effects of insulin/IGF-1 and counteregulatory effects of angiotensin II type 1 receptor (AT1R) and mineralocorticoid receptor (MR) activation in endothelial cells. Insulin actions on the blood vessel are partially mediated by an increased production of Nitric Oxide (NO) through phosphorylation and secondary activation of endothelial nitric oxide synthase (eNOS). AT1R activation decreases the availability of NO via induction of insulin resistance, diminishing eNOS mRNA stability and promoting NADPH oxidase-induced reactive
Figure 1B) Opposing effects of Angiotensin II and aldosterone vs. insulin/IGF-1 on vascular smooth muscle cells (VSMC). Insulin and IGF-1 cause VSMC relaxation while Ang II and mineralocorticoids cause contraction.
chain; MLCK, myosin light chain kinase; MR, mineralocorticoid receptor; Na/Ca exch, sodium/calcium exchanger; Na pump, sodium pump; NADPH oxidase, reduced nicotinamide adenine dinucleotide phosphate; NOX2, catalytic subunit of NADPH oxidase; p22, p47, p40, p67, subunits NADPH oxidase; PH, pleckstrin homology domain; PI3K, phosphatidylinositol (PI) 3-kinase; PIP2, Phosphatidylinositol bisphosphate; PIP3, Phosphatidylinositol (3,4,5)-trisphosphate; Rac, Small G protein; RhoA, small GTPase; ROK, Rho kinase; Ser473, serine 473; SOD, superoxide dismutase; Thr308, threonine 308.

**Figure 2: Functional and metabolic effects of insulin and IGF-1 in the heart.** Insulin and IGF-1 modulate glucose transport, glycogen synthesis, lipid metabolism, growth, contractility and apoptosis in cardiomyocytes. Upon activation of the mitogen-activated protein kinase (MAPK) pathway, insulin/IGF-1 and angiotensin II/ aldosterone signaling may converge to cause deleterious effects on cardiovascular tissue.
Figure 3: Angiotensin II and aldosterone/corticosterone antagonism to metabolic actions of insulin/IGF-1 in skeletal muscle. AT$_1$R activation impaired insulin signaling in skeletal muscle with consequent reduction in glucose uptake. Possible mechanisms involved in skeletal muscle insulin resistance include inadequate interaction between insulin receptor and IRS, serine phosphorylation of IRS, lack of phosphorylation of key tyrosine residues in IRS, and reduced activation of Akt. Through activation of NADPH oxidase, aldosterone can increase oxidative stress and impair metabolic insulin signaling.

Akt, protein kinase B; AT$_1$R, Angiotensin II type 1 receptor; GDP, guanosine diphosphate; ERK, extracellular signal-regulated kinase; GLUT4, glucose transporter 4. GRE, glucocorticoid response element; GTP, guanosine triphosphate; Gq, G protein alpha q subunit; IRS, Insulin receptor substrate; JAK, Janus kinase; JNK, c-Jun N-terminal kinase; MAP, mitogen-activated protein; MEK, extracellular signal-regulated kinase; MEKK-1, mitogen-activated protein kinase/ERK kinase kinase 1; MR, mineralocorticoid receptor; MTOR, mammalian target of rapamycin; NO, nitric oxide; NOS, NO synthase; NOX2, catalytic subunit of NADPH oxidase; NADPH oxidase, reduced nicotinamide adenine dinucleotide phosphate; O$_2^-$, superoxide; ONOO$^-$, peroxinitrite; p22, p47, p40, p67, subunits NADPH oxidase; PKCz, protein kinase C zeta; PDK1, phosphoinositide-dependent kinase 1; PH, pleckstrin homology domain; PI3K, phosphatidylinositol (PI) 3-kinase; PIP2, Phosphatidylinositol bisphosphate; PIP3, Phosphatidylinositol (3,4,5)-trisphosphate; Rac, small G protein; Raf-1, serine/threonine-specific kinase; Ras, small GTPase; RhoA, small GTPase; ROK, Rho kinase; Ser307, serine307; Ser473, serine 473; Ser 616, serine 616; SOD, superoxide dismutase; STAT; signal transducer and activator of transcription; Tyr612, tyrosine 612; tyr632, tyrosine 632; Thr308, threonine 308.
Figure 4: Activation of the Renin-Angiotensin-Aldosterone System induces vascular oxidative stress and insulin resistance. Vascular superoxide (O$_2^-$) generation as detected by Dihydroethidium (DHE) immunostaining in the insulin resistant transgenic TG(mRen2)27 rat (Ren2), which overexpresses the renin gene with subsequent elevated tissue levels of Ang II relative to Sprague-Dawley (SD) control. Intracellular O$_2^-$ converts DHE to ethidium which binds to double-stranded DNA resulting in nuclear red fluorescence. The green autofluorescence is specific for elastin fibers.

Figure 5: Micro-positron emission tomograph (PET) and electrocardiographically gated magnetic resonance images (MRI) of Sprague-Dawley animals with and without insulin/glucose stimulation. Rats are supine and the images are coronal views. Upon insulin/glucose stimulation there is increased myocardial glucose uptake (noted by increased brightness) when compared with the basal state.

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Figure 1

A:

VSMC

Endothelial Cells

B:
Figure 2:
Figure 3

Cardiac Muscle

Aldosterone
MR
NADPH Oxidase
AT,R

Ang II

IGF-I
IGF-I receptor
shc
crk
RAS

Insulin
IRS-1

MAP Kinase
p38, JNK 1, ERK1/2

NF-κβ, AP-1, HIF-1, Erg1

Transcriptional changes

DNA Synthesis
Mitogenesis

Translational changes

Contractility
Anti-apoptosis
Glucose Transport

Copyright Information
Figure 4

SD

Ren2

SD

Ren2
Figure 5

$^{18}$F-FDG Cardiac Imaging

Control

Micro-PET™

Gated MRI

Insulin/Glucose Treated

Micro-PET™