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## Metabolic Actions of Angiotensin II and Insulin: A Microvascular Endothelial Balancing Act

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### Abstract

Metabolic actions of insulin to promote glucose disposal are augmented by nitric oxide (NO)-dependent increases in microvascular blood flow to skeletal muscle. The balance between NO-dependent vasodilator actions and endothelin-1-dependent vasoconstrictor actions of insulin is regulated by phosphatidylinositol 3-kinase-dependent (PI3K) - and mitogen-activated protein kinase (MAPK)-dependent signaling in vascular endothelium, respectively. Angiotensin II acting on AT<sub>2</sub> receptor increases capillary blood flow to increase insulin-mediated glucose disposal. In contrast, AT<sub>1</sub> receptor activation leads to reduced NO bioavailability, impaired insulin signaling, vasoconstriction, and insulin resistance. Insulin-resistant states are characterized by dysregulated local renin-angiotensin-aldosterone system (RAAS). Under insulin-resistant conditions, pathway-specific impairment in PI3K-dependent signaling may cause imbalance between production of NO and secretion of endothelin-1, leading to decreased blood flow, which worsens insulin resistance. Similarly, excess AT<sub>1</sub> receptor activity in the microvasculature may selectively impair vasodilation while simultaneously potentiating the vasoconstrictor actions of insulin. Therapeutic interventions that target pathway-selective impairment in insulin signaling and the imbalance in AT<sub>1</sub> and AT<sub>2</sub> receptor signaling in microvascular endothelium may simultaneously ameliorate endothelial dysfunction and insulin resistance. In the present review, we discuss molecular mechanisms in the endothelium underlying microvascular and metabolic actions of insulin and Angiotensin II, the mechanistic basis for microvascular endothelial dysfunction and insulin resistance in RAAS dysregulated clinical states, and the rationale for therapeutic strategies that restore the balance in vasodilator and constrictor actions of insulin and Angiotensin II in the microvasculature.

### Keywords

Nitric Oxide; Insulin Resistance; Endothelial Dysfunction; Angiotensin II

### 1. Introduction

Insulin resistance is frequently present in obesity, hypertension, coronary artery disease, dyslipidemias, and metabolic syndrome (DeFronzo and Ferrannini, 1991, Petersen, Dufour,

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Savage et al., 2007). Insulin regulates glucose homeostasis by promoting glucose disposal in skeletal muscle and adipose tissue (Petersen et al., 2007). In addition to its direct actions on the skeletal muscle, insulin regulates nutrient delivery to target tissues by actions on microvasculature (Baron and Clark, 1997, Clark, 2008, Clark, Colquhoun, Rattigan et al., 1995, Clark, Wallis, Barrett et al., 2003, Barrett, Wang, Upchurch et al., 2011). These vasodilator actions of insulin are nitric oxide (NO)-dependent and lead to increased skeletal muscle microvascular perfusion that further enhances glucose uptake in skeletal muscle (Muniyappa, Montagnani, Koh et al., 2007, Vicent, Ilany, Kondo et al., 2003, Vincent, Clerk, Lindner et al., 2004, Zhang, Vincent, Richards et al., 2004). These actions of insulin on skeletal muscle microvasculature appear to be a rate limiting step for insulin-mediated glucose disposal. At the cellular level, balance between phosphatidylinositol 3-kinase-(PI3K)-dependent insulin-signaling pathways that regulate endothelial NO production and mitogen activated protein kinase (MAPK)-dependent insulin-signaling pathways regulating the secretion of the vasoconstrictor endothelin-1 (ET-1) determines the microvascular response to insulin. Insulin resistance is typically defined as decreased sensitivity or responsiveness to metabolic actions of insulin such as insulin-mediated glucose disposal. However, diminished sensitivity to the vascular actions of insulin also plays an important role in the pathophysiology of insulin-resistant states (Natali, Taddei, Quinones Galvan et al., 1997, Baron, Laakso, Brechtel et al., 1991). Endothelial insulin resistance is typically accompanied by reduced PI3K-NO pathway and an intact or heightened MAPK-ET1 pathway (Muniyappa et al., 2007).

The Renin-angiotensin-aldosterone system (RAAS) plays a major role in microvascular function and remodeling. Ang II regulates endothelial NO production, arterial tone, skeletal muscle microvascular perfusion, and glucose metabolism in a receptor (AR)-specific manner (AT<sub>1</sub>R vs. AT<sub>2</sub>R) (Chai, Wang, Dong et al., 2011, Chai, Wang, Liu et al., 2010). In contrast to AT<sub>1</sub>R, stimulation of AT<sub>2</sub>R increases NO production, reduces vascular tone, and augments skeletal muscle microvascular perfusion (Chai et al., 2011, Chai et al., 2010). Activation of RAAS in insulin-sensitive tissues is known to induce insulin resistance (Cooper, Whaley-Connell, Habibi et al., 2007, Lastra-Lastra, Sowers, Restrepo-Erazo et al., 2009). In particular, chronic activation of RAAS impairs insulin signaling, increases oxidative stress, and reduces NO bioavailability (Cooper et al., 2007). However, insulin resistance also increases local RAAS activity triggering a vicious cycle that leads to endothelial dysfunction, atherosclerosis, inflammation, and dysmetabolic states associated with obesity, diabetes, and hypertension. Thus, the relative contributions of AT<sub>1</sub>R and AT<sub>2</sub>R activation and cross-talk between the signaling pathways of insulin and Ang II appear to modulate endothelial function. Herein, we discuss the cellular mechanisms and signaling pathways underlying the microvascular actions of insulin and Ang II, the metabolic consequences of an imbalance in these pathways, and potential therapeutic interventions that may improve microvascular function in insulin-resistant conditions.

## 2. Role of Skeletal Muscle Microvasculature in Metabolism

Arterioles, capillaries and venules that are less than 150  $\mu\text{m}$  in diameter are generally termed “microvascular” (Segal, 2005). Microvascular perfusion, especially in insulin sensitive tissues such as skeletal muscle and adipose tissue plays an important role in providing adequate delivery of nutrients, capillary surface area for nutrient exchange, and increased vascular permeability for nutrient transfer from plasma to tissue interstitium (Baron and Clark, 1997, Clark, 2008, Clark et al., 1995, Clark et al., 2003). The rate of substrate extraction and hence it’s arterio-venous difference (A–V) is governed by the classic equation:  $[(V)-(A)]=[(I)-(A)]\times[1-e^{-PS/Q}]$ , where “V” is the venous, “A” the arterial, and “I” is the interstitial substrate concentration, “P” is surface permeability, “S” is exchange surface area, and “Q” is the plasma flow rate (Renkin and Rosell, 1962). Thus it is evident

that a change in surface area and/or permeability significantly influences tissue nutrient extraction (Baron, Tarshoby, Hook et al., 2000). Skeletal muscle capillaries are arranged in groups termed 'microvascular units'; recruitment and perfusion of these units are determined by the tone of capillary sphincter of terminal arteriole. Relaxation of the capillary sphincter dilates terminal arteriole and recruits capillaries to further increase nutrient exchange surface area and substrate extraction by the skeletal muscle (Segal, 2005). Dilation and constriction of terminal arterioles is modulated by endothelium-derived vasoactive substances that include vasodilators (e.g., nitric oxide (NO), prostaglandins (PGI<sub>2</sub>), endothelium-derived hyperpolarization factor (EDHF), epoxyeicosatrienoic acids (EETs)) and vasoconstrictors (e.g., endothelin-1 (ET-1), prostanoids, isoprostanes). Thus, the balance in actions of vasodilators and vasoconstrictors determine microvascular blood volume and perfusion.

### 3. Endothelium Derived Vasodilatory and Vasoconstrictor Factors

#### 3.1 Nitric Oxide

In the vasculature, NO, a short-lived signal molecule is released in a transient fashion on demand by enzymatic activation of a constitutively present NO synthase (NOS) in the endothelium (eNOS) (Michel and Feron, 1997). Significant amounts of NO can also be produced in a sustained fashion in endothelium and vascular smooth muscle cells (VSMCs) in response to various agents and cytokines, through the expression of the inducible NOS (iNOS) (Michel and Feron, 1997). NO released from either source decreases vascular tone, inhibits VSMC proliferation, induces apoptosis, attenuates platelet aggregation, and reduces cell adhesion to vascular walls. Vasodilatory actions of NO are primarily mediated via reductions in VSMC intracellular calcium (Ca<sup>2+</sup>) concentrations secondary to NO-mediated guanylate cyclase activation and cGMP formation (Michel and Feron, 1997, Michel and Vanhoutte, 2010). eNOS is a homodimer that converts L-arginine and O<sub>2</sub> to L-citrulline and NO. eNOS resides in the caveolae and bound to the caveolar protein, caveolin-1 that inhibits its activity (Dessy, Feron and Balligand, 2010, Duran, Breslin and Sanchez, 2010). Elevations in cytoplasmic Ca<sup>2+</sup> promote binding of calmodulin to eNOS that subsequently displaces caveolin and activates eNOS (Dessy et al., 2010, Duran et al., 2010). In addition to undergoing regulatory posttranslational modifications, phosphorylation of various amino acid residues regulates eNOS activity (Michel and Vanhoutte, 2010). Phosphorylation of Ser<sup>1177</sup> reduces Ca<sup>2+</sup> sensitivity of eNOS such that NO production is triggered at ambient intracellular Ca<sup>2+</sup> levels. However, phosphorylation at Thr<sup>495</sup> prevents calmodulin binding to eNOS to reduce NO production (Michel and Vanhoutte, 2010, Fleming, 2010). Similarly, tyrosine phosphorylation at Tyr<sup>657</sup> results in decreased eNOS activity (Michel and Vanhoutte, 2010, Fleming, 2010). In addition to changes in phosphorylation status of the enzyme, alterations in intracellular levels of tetrahydrobiopterin (BH<sub>4</sub>), an essential co-factor or the substrate L-arginine can also modulate enzyme activity (Crabtree and Channon, 2011, Gielis, Lin, Wingler et al., 2011).

#### 3.2 Prostanoids/Eicosanoids

Prostanoids are the cyclooxygenase metabolites of arachidonic acid and include prostaglandins (PGD<sub>2</sub>, PGE<sub>2</sub>, PGF<sub>2</sub>, PGI<sub>2</sub>), and thromboxane (TxA<sub>2</sub>) (Bos, Richel, Ritsema et al., 2004). Agonist stimulation of endothelial cells results in increases in cytosolic Ca<sup>2+</sup> concentration that subsequently activates phospholipase A<sub>2</sub> (PLA<sub>2</sub>) (Tang, Leung, Huang et al., 2007). PLA<sub>2</sub> acts on cell membrane phospholipids to release arachidonic acid that is metabolized by cyclooxygenases (COX): COX-1 is expressed constitutively in human endothelial cells and endothelial COX-2 is induced following exposure to inflammatory stimuli and agents (Garavito and DeWitt, 1999, Vane, Bakhle and Botting, 1998). However, COX-1 is the principal COX that produces endoperoxides (PGH<sub>2</sub>) in healthy and abnormal endothelium. Specific synthases convert PGH<sub>2</sub> to prostanoids,

PGD<sub>2</sub>, PGE<sub>2</sub>, PGF<sub>2</sub>, PGI<sub>2</sub>, or TxA<sub>2</sub> (Davidge, 2001). Endothelial prostanoids diffuse into VSMCs to interact with specific G-protein-coupled membrane receptors. PGI<sub>2</sub> and TxA<sub>2</sub> bind to VSMC prostacyclin receptor (IP) and thromboxane prostanoid receptor (TP), respectively. IP receptor activation in VSMC leads to intracellular increases in cAMP that leads to vasorelaxation (Bos et al., 2004). In contrast, TP receptor activation in VSMC leads to increases in intracellular Ca<sup>2+</sup> and vasoconstriction (Bos et al., 2004). Reactive oxygen species (ROS) are also known to activate COX (Harlan and Callahan, 1984). Arachidonic acid is also metabolized by cytochrome P450 (CYP) epoxygenase to produce epoxyeicosatrienoic acids (EETs) (Pfister, Gauthier and Campbell, 2010). Acting as endothelium-derived hyperpolarizing factors (EDHFs), EETs act locally to decrease vascular tone. In VSMC, EETs activates large conductance, calcium-sensitive potassium (BK<sub>Ca</sub>) channels, leading to vasorelaxation (Pfister et al., 2010). Furthermore, EETs activate cation channels of the transient receptor potential gene family (TRPV4) to augment Ca<sup>2+</sup> signaling in the endothelium that leads to NO formation (Baylie and Brayden, 2011). Thus, the relative amounts and activities of PGI<sub>2</sub>, TxA<sub>2</sub>, and EETs play an important role in the regulation of microvascular tone (Feletou, Huang and Vanhoutte, 2010).

### 3.3 Endothelin-1

ET-1 by its actions on VSMC tone, oxidative stress, proliferation, and apoptosis plays an important role in vascular function (Marasciulo, Montagnani and Potenza, 2006, Khimji and Rockey, 2010). ET-1 secretion in the endothelium is regulated by two distinct pathways: a constitutive pathway, determined by gene transcription and a regulated pathway involving stimulated release from preformed ET-1 precursors in intracellular storage granules (Marasciulo et al., 2006, Khimji and Rockey, 2010, Goel, Su, Flavahan et al., 2010). ET-1 is synthesized predominantly in vascular endothelial cells, where the endothelin gene product, proET-1 (ppET-1) is sequentially converted to big-ET-1 (bET-1) and active ET-1 by proteases (Marasciulo et al., 2006, Khimji and Rockey, 2010). Ang II and insulin increase (Hsu, Chen, Chang et al., 2004, Hu, Levin, Pedram et al., 1993, Kobayashi, Nogami, Taguchi et al., 2008, Oliver, de la Rubia, Feener et al., 1991), while NO and PGI<sub>2</sub> inhibit ppET-1 expression in the endothelium (Bourque, Davidge and Adams, 2011, Prins, Hu, Nazario et al., 1994). Locally released ET-1, acting in a paracrine fashion binds to ET<sub>A</sub> receptors on VSMC causing sustained vasoconstriction (Verhaar, Strachan, Newby et al., 1998).

## 4. Effects of Insulin on Endothelium Derived Vasodilatory and Vasoconstrictor Factors

### 4.1 Insulin-Stimulated Signaling Pathways Mediating NO Production in Endothelium

Insulin signaling pathways in endothelium regulating production of NO have been well characterized, primarily in endothelial cells in culture (Fig. 1). This pathway begins proximally with insulin binding to its receptor and culminates in the phosphorylation and activation of endothelial NO synthase (eNOS). Physiological concentrations of insulin (100 – 500 pM) can selectively activate ~ 40,000 insulin receptors (IR) on human endothelial cell surface per cell to increase NO production (Zeng and Quon, 1996). However, this response is absent in human umbilical vein endothelial cells (HUVEC) transfected with mutant kinase-deficient insulin receptors (Zeng, Nystrom, Ravichandran et al., 2000). Thus, increase in insulin receptor tyrosine kinase activity is essential and results in phosphorylation of IRS-1 which then binds and activates PI3K (Zeng et al., 2000). Lipid products of PI3K (PI-3,4,5-triphosphate (PIP<sub>3</sub>)) stimulate phosphorylation and activation of PDK-1 that in turn phosphorylates and activates Akt (Montagnani, Ravichandran, Chen et al., 2002). Akt directly phosphorylates eNOS at Ser<sup>1177</sup> resulting in increased eNOS activity and subsequent NO production (Montagnani et al., 2002). The calcium chelator BAPTA

does not inhibit the ability of insulin to stimulate phosphorylation of eNOS at Ser<sup>1177</sup>, suggesting that insulin-induced eNOS activation is calcium-independent (Montagnani et al., 2002). Akt-mediated phosphorylation of eNOS alone is not sufficient for NO production. eNOS activity is critically controlled by calmodulin (CaM) and by binding of the molecular chaperone heat-shock protein 90 (HSP90). Insulin stimulates calmodulin and HSP90 binding to eNOS which facilitates insulin-stimulated phosphorylation and activation of eNOS at Ser<sup>1177</sup> by Akt (Takahashi and Mendelsohn, 2003). The Ras/MAP-kinase branch of insulin-signaling pathways does not contribute significantly to activation of eNOS in response to insulin. However, inhibition of MAPK-dependent insulin signaling pathway may enhance the PI3K-dependent vascular actions of insulin on eNOS (Montagnani, Golovchenko, Kim et al., 2002). This suggests that the MAPK pathway negatively regulates the PI3K pathway.

#### 4.2 Insulin-Stimulated Signaling Pathways Mediating ET-1 Production in Endothelium

The MAPK-dependent signaling pathways regulates insulin's effects related to mitogenesis, growth, and differentiation. However, this pathway plays a key role in the upregulating endothelial gene expression and secretion of ET-1, a potent vasoconstrictor (Fig. 1). This signaling branch 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 Sos. This activates the small GTP binding protein Ras, which then initiates a kinase phosphorylation cascade involving Raf, MAP-kinase/extracellular signal-regulated kinase (MEK), and MAP-kinase (MAPK) (Nystrom and Quon, 1999, Taniguchi, Emanuelli and Kahn, 2006). Although the precise transcription factors mediating insulin-stimulated expression of ET-1 are not known, an insulin-responsive element in the ET-1 gene has been identified (Oliver et al., 1991). Nevertheless, insulin exposure stimulates ET-1 secretion from the endothelium that peaks ~ 15–20 min that subsequently leads to sustained vasoconstriction (Hu et al., 1993).

#### 4.3 Insulin-Stimulation of PGI<sub>2</sub> and EDHF

Insulin acutely stimulates production of PGI<sub>2</sub> from vascular endothelium (Sobrevia, Nadal, Yudilevich et al., 1996). NO can directly suppress activity of COX-1 and decrease both basal and stimulated release of PGI<sub>2</sub> (Osanai, Fujita, Fujiwara et al., 2000). Inhibition of insulin-stimulated NO production using N (G)-nitro-L-arginine methyl ester (L-NAME) does not prevent a PGI<sub>2</sub> production in endothelial cells. This suggests that insulin has direct actions to stimulate PGI<sub>2</sub> production in an NO-independent fashion (Sobrevia et al., 1996). However, insulin-signaling pathways regulating PGI<sub>2</sub> production are yet to be elucidated. In addition to PGI<sub>2</sub>, insulin augments EDHF-evoked hyperpolarization in arterioles (Imaeda, Okayama, Okouchi et al., 2004). Interestingly, PLA<sub>2</sub>, insulin receptor, and caveolin-1 are co-localized in the caveolae and this spatial arrangement is essential for phospholipase A<sub>2</sub>-dependent EDHF formation (Graziani, Bricko, Carmignani et al., 2004, Wang, Wang and Barrett, 2011). Thus, the balance in NO, PGI<sub>2</sub>, EDHF, and ET-1 actions determines insulin's actions on vascular tone.

### 5. Effects of Angiotensin II on Endothelium Derived Vasodilatory and Vasoconstrictor Factors

The vascular effects of Ang II are mediated by the activation of two heptahelical G-protein coupled membrane receptors: Ang II type 1 receptor (AT<sub>1</sub>R) and type 2 (AT<sub>2</sub>R) receptor. The vasoconstrictive, hypertensive, and proliferative actions of Ang II are mediated by AT<sub>1</sub>R while AT<sub>2</sub>R activation has been suggested to counteract these effects (Mehta and Griendling, 2007, Watanabe, Barker and Berk, 2005, Lemarie and Schiffrin, 2010). Both AT<sub>1</sub>R and AT<sub>2</sub>R are expressed in endothelial cells; AT<sub>2</sub>R expression decreases following birth but is upregulated in the vasculature in pathological states. Thus, relative proportions

of AT<sub>1</sub>R and AT<sub>2</sub>R in the endothelium may determine the ultimate vascular effects of Ang II (Watanabe et al., 2005, Lemarie and Schiffrin, 2010). Both AT receptors activate a wide variety of downstream signaling molecules that have been extensively characterized in VSMCs (Mehta and Griendling, 2007). However, signaling pathways mediating AT<sub>1</sub>R actions in endothelial cells are increasingly the focus of recent studies. This review will be confined to studies in the endothelium.

### 5.1 Signaling Pathways Mediating AT<sub>1</sub>R Activation in Endothelium

AT<sub>1</sub>R interacts with multiple heterotrimeric G proteins, including G<sub>q/11</sub>, G<sub>12</sub>, G<sub>13</sub>, and G<sub>i</sub> to produce signaling molecules such as inositol triphosphate, diacylglycerol, reactive oxygen species (ROS), and NO (Mehta and Griendling, 2007, Garrido and Griendling, 2009). It activates receptor tyrosine kinases (PDGF, EGFR, insulin receptor), non-receptor tyrosine kinases [Janus kinases, focal adhesion kinase, Ca<sup>2+</sup>-dependent proline-rich tyrosine kinase (Pyk2), c-Src family kinases], and serine–threonine kinases (MAPK, mitogen-activated protein kinase; ERK, extracellular-signal-regulated kinase; PKC, protein kinase C; and JNK, c-Jun N terminal kinase) (Mehta and Griendling, 2007, Touyz and Schiffrin, 2000). Many of these signaling molecules mediate AT<sub>1</sub>R-induced impairment in the release and action of endothelium-derived relaxing factors and potentiation of vasoconstrictors.

### 5.2 AT<sub>1</sub> Receptor, eNOS, and NO Bioavailability

Short-term stimulation of AT<sub>1</sub>R activates eNOS while chronic stimulation suppresses eNOS activity and reduces NO bioavailability (Cai, Li, Dikalov et al., 2002, Imanishi, Kobayashi, Kuroi et al., 2006) (Fig. 2). The magnitude of NADPH oxidase mediated ROS production has been proposed to explicate these apparently discrepant actions. The mechanism(s) of NADPH oxidase activation by Ang II are complex and unclear. A functional NADPH oxidase is a multimeric enzyme that consists of NOX2, p22phox, and cytosolic subunits (p47phox, p67phox, and small G-protein, Rac) (Garrido and Griendling, 2009). Upon AT<sub>1</sub>R activation in the endothelium, caveolin-1 recruits cytosolic p47phox to caveolae to produce a functional enzyme (Lobysheva, Rath, Sekkali et al., 2011). The formation of ROS and H<sub>2</sub>O<sub>2</sub> following NADPH activation is associated with recruitment of active c-Src and Abl to the caveolae where eNOS resides (Cai et al., 2002, Lobysheva et al., 2011). c-Src in a PI3K-dependent fashion acutely stimulates eNOS to produce NO (Fulton, Church, Ruan et al., 2005). In addition to mediating the acute NO stimulatory effects of Ang II, Src tyrosine kinase- also facilitates chronic Ang II-induced eNOS protein expression (Li, Lerea, Li et al., 2004). In addition to c-Src, Ang II activates sphingosine kinase to increase sphingosine-1-phosphate (S1P) which then activates eNOS through the S1P receptor (Mulders, Hendriks-Balk, Mathy et al., 2006). Sphingosine kinase activates eNOS through the release of intracellular Ca<sup>2+</sup> and phosphorylation of Akt and eNOS via the PI3K pathway (Mulders et al., 2006). Ang II has also been shown to induce NO production through G<sub>q</sub>-dependent eNOS phosphorylation at Ser1177 (Suzuki, Eguchi, Ohtsu et al., 2006). However, these transient NO stimulatory effects of Ang II are overwhelmed by the oxidative stress that ensues with chronic Ang II stimulation of AT<sub>1</sub>R and accompanying NADPH oxidase activation.

Chronic Ang II stimulation results in oxidative stress due to sustained and excess ROS production and reduction of anti-oxidative enzymes such as superoxide dismutase (SOD) which catalyzes the dismutation of O<sup>2-</sup> into oxygen and hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>). This increased oxidative stress may reduce NO bioavailability by multiple mechanisms (Fig. 2). First, oxidative stress and Ang II stimulates PYK2 that subsequently inactivates NOS by phosphorylating Tyr<sup>657</sup> in the reductase domain of the enzyme (Loot, Schreiber, Fisslthaler et al., 2009). Second, Ang II-induced oxidative stress reduces intracellular levels of essential eNOS cofactor, BH<sub>4</sub> secondary to ROS mediated oxidation of BH<sub>4</sub> and reduced levels of

guanosine triphosphate cyclohydrolase I (GTPCH), the rate-limiting enzyme for BH<sub>4</sub> synthesis and dihydrofolate reductase (DHFR), the enzyme mediating BH<sub>4</sub> regeneration (Crabtree and Channon, 2011, Chalupsky and Cai, 2005). This leads to eNOS “uncoupling” where in the native eNOS dimer is uncoupled to monomers that produce significant amounts of ROS instead of NO (Gielis et al., 2011). This sets up a self-perpetuating vicious cycle leading to further reduction in NO activity and accentuated oxidative stress. Third, excess ROS reacts with NO to produce peroxynitrite that can oxidize various key intracellular proteins including eNOS to cause endothelial dysfunction (Pueyo, Arnal, Rami et al., 1998, Wattanapitayakul, Weinstein, Holycross et al., 2000). Fourth, chronic Ang II increases microvascular levels of asymmetrical dimethylarginine (ADMA), an endogenous inhibitor of eNOS (Wang, Luo, Wang et al., 2010). In the endothelium, ADMA-degrading enzyme dimethylarginine dimethylaminohydrolase (DDAH) is redox-sensitive (Lin, Ito, Asagami et al., 2002). Chronic Ang II-induced oxidative stress reduces microvascular DDAH expression leading to elevated ADMA levels and reduced NO bioavailability (Wang et al., 2010). Finally, Ang II increases endothelial arginase activity/expression through G<sub>12/13</sub> coupled to AT<sub>1</sub>R and subsequent activation of RhoA/ROCK/p38 MAPK pathway (Shatanawi, Romero, Iddings et al., 2011). Due to colocalization of eNOS and arginase in the caveolae, increased arginase activity reduces ambient L-arginine concentrations, a key eNOS substrate. The combination of reduced substrate availability and elevations in ADMA may substantially reduce eNOS activity and NO bioavailability.

### 5.3 AT<sub>1</sub> Receptor and Endothelium Derived Vasoconstrictors

Ang II stimulates endothelial ET-1 production through AT<sub>1</sub>R-mediated mobilization of intracellular Ca<sup>2+</sup> and activation of PKC. PKC-dependent activation of the transcription factor, AP-1 that mediates preproendothelin-1 mRNA transcription (Imai, Hirata, Emori et al., 1992, Chua, Chua, Diglio et al., 1993, Stow, Jacobs, Wingo et al., 2011). Similarly, Ang II induced ROS also activates the Ras/Raf/ERK/AP-1 pathway to upregulate endothelial ET-1 gene expression (Hsu et al., 2004) (Fig. 2). Due to the tonic inhibition of ET-1 by NO, Ang II-induced reduced NO bioavailability NO augments basal and agonist-stimulated ET-1 production in endothelial cells (Bourque et al., 2011). Furthermore, ET-1 enhances endothelial angiotensin converting enzyme (ACE) activity leading to enhanced generation of Ang II (Kawaguchi, Sawa and Yasuda, 1991). Moreover, ET-1 mimics many of the actions of Ang II to reduce NO bioavailability and cause endothelial dysfunction (Bourque et al., 2011). Thus, upregulation of local Ang II and ET-1 generates a self-perpetuating cycle that results in significant microvascular dysfunction.

COX-derived prostanoids and eicosanoids play an important role in regulation of vascular tone. Ang II increases COX expression in human endothelial cells in culture (Li Volti, Seta, Schwartzman et al., 2003). Chronic Ang II infusion in mice also increases endothelial COX-1 expression and activity in a ROS-dependent manner (Viridis, Colucci, Fornai et al., 2007). Increased COX-1 activity produces a contracting prostanoid, to activate VSMC TP receptors. These findings are consistent with other studies that show COX-derived vasoconstrictors mediate Ang II –induced endothelial dysfunction in an AT<sub>1</sub>R-dependent manner (Kane, Etienne-Selloum, Madeira et al., 2010, Capone, Faraco, Anrather et al., 2010, Matsumoto, Ishida, Nakayama et al., 2010, Alvarez, Perez-Giron, Hernandez et al., 2007). Ang II increases production of the prostanoid vasodilator, PGI<sub>2</sub> (Nie, Wu, Zhang et al., 2006). However, during states of oxidative stress, peroxynitrite formed due to uncoupled eNOS inactivates PGI<sub>2</sub> synthase (PGIS) by nitration (Nie et al., 2006). The resulting imbalance shifts Ang II-stimulated PGI<sub>2</sub>-dependent relaxation into a persistent vasoconstriction. Similarly, endothelium-derived EETs are vasodilators. Microvascular constrictor responses to Ang II are augmented by CYP epoxygenase inhibition suggesting that the metabolites of the epoxygenase pathway play an important role in modulating

microvascular tone (Imig and Deichmann, 1997). Chronic treatment of endothelial cells with Ang II upregulates soluble epoxide hydrolase (sEH), an enzyme that degrades EETs in the vascular wall (Ai, Fu, Guo et al., 2007). This transcriptional upregulation of endothelial sEH is mediated by AT<sub>1</sub>R activation of the transcription factor AP-1 (Ai et al., 2007). Increased sEH enhances the hydrolysis of the vasodilatory EETs in favor of enhanced vasoconstriction in metabolic states characterized by increased vascular Ang II activity. Thus, Ang II activation of AT<sub>1</sub>R leads to oxidative stress, reduced NO bioavailability, and enhanced secretion and action of ET-1 and endothelium-derived vasoconstrictor prostanoids.

#### 5.4 AT<sub>2</sub> Receptor, eNOS, and NO Bioavailability

AT<sub>2</sub>R activation leads to vasodilation and counteracts the vasoconstrictive effects of AT<sub>1</sub>R stimulation (Lemarie and Schiffrin, 2010, Batenburg, Garrelts, Bernasconi et al., 2004, Siragy, Inagami, Ichiki et al., 1999, Bosnyak, Welungoda, Hallberg et al., 2010) (Fig. 2). In the presence of AT<sub>1</sub>R blocker, Ang II augments vasodilation that is sensitive to inhibition of AT<sub>2</sub>R, bradykinin B<sub>2</sub> receptor (B<sub>2</sub>R), and eNOS (Batenburg et al., 2004, Carey, 2005, Cosentino, Savoia, De Paolis et al., 2005, Gohlke, Pees and Unger, 1998, Katada and Majima, 2002, Seyedi, Xu, Nasjletti et al., 1995, Tsutsumi, Matsubara, Masaki et al., 1999). AT<sub>2</sub>R activation increases intracellular acidosis, stimulates kininogenases, and elevates bradykinin formation in the endothelium (Tsutsumi et al., 1999). Bradykinin then binds to B<sub>2</sub>R to activate eNOS and NO formation. Prolylcarboxypeptidase is a plasma prekallikrein activator in endothelial cells that increases plasma kallikrein (Shariat-Madar, Mahdi and Schmaier, 2002). Plasma kallikrein cleaves high-molecular-weight kininogen to liberate kinins such as bradykinin (Zhu, Carretero, Liao et al., 2010). Activation or overexpression of AT<sub>2</sub>R in the endothelium increases prolylcarboxypeptidase activity that leads to bradykinin formation (Zhu et al., 2010). Vasodilatory actions of Ang II are thus more pronounced in conditions characterized by increased AT<sub>2</sub>R expression. In addition to antagonizing AT<sub>1</sub>R-mediated vasoconstrictor actions through enhanced NO release, AT<sub>2</sub>R form homo- or hetero-dimers with AT<sub>1</sub>R. Dimerization of AT<sub>2</sub>R with AT<sub>1</sub>R or B<sub>2</sub>R, enhances vasodilation (Abadir, Periasamy, Carey et al., 2006, AbdAlla, Lother, Abdel-tawab et al., 2001). Thus, the relative activity and endothelial expression of AT<sub>1</sub>R and AT<sub>2</sub>R shape the magnitude of vasodilator response to Ang II.

## 6. Effects of Insulin and Angiotensin II on Skeletal Muscle Microvasculature and Glucose Disposal

### 6.1 Insulin-stimulated skeletal muscle capillary recruitment and glucose disposal

As discussed previously, PI3K-dependent insulin signaling pathways in vascular endothelium, skeletal muscle, and adipose tissue regulate vasodilator and metabolic actions of insulin. However, MAPK-dependent insulin signaling pathways tend to promote pro-hypertensive and pro-atherogenic actions of insulin in various tissues. In humans, intravenous insulin infusion stimulates capillary recruitment, vasodilation, and increased blood flow in an NO-dependent fashion (Vincent et al., 2004). In the initial few minutes of insulin exposure, dilation of terminal arterioles increases the number of perfused capillaries (capillary recruitment). This is followed by relaxation of larger resistance vessels that increases overall limb blood flow (maximum flow reached after 2 h) (Baron and Clark, 1997). Increasing arterial plasma insulin levels to ~ 300 pM in human forearm results in a 25% increase in capillary blood volume in the deep flexor muscles (Coggins, Lindner, Rattigan et al., 2001). Similarly, in a more physiologic setting, microvascular volume in human forearm increases by ~ 45% an hour after a mixed meal (Vincent, Clerk, Lindner et al., 2006). Thus, after a mixed meal, an oral glucose load, or infusion of insulin, recruitment of capillaries expands the capillary surface area and increases muscle blood flow which together substantially increase glucose and insulin delivery. Inhibitors of NOS that block



insulin-mediated capillary recruitment cause a concomitant 40% reduction in glucose disposal (Vicent et al., 2003, Vincent et al., 2004). Thus, PI3K-dependent vascular actions of insulin directly promote glucose uptake in skeletal muscle.

## 6.2 Angiotensin II, skeletal muscle capillary recruitment, and glucose disposal

The effects of Ang II on human microcirculation has not been studied. Local infusions of Ang II increase oxygen uptake and glucose metabolism in rat skeletal muscle (Chai et al., 2010, Zhang, Newman, Richards et al., 2005, Rattigan, Dora, Tong et al., 1996, Newman, Rattigan and Clark, 2002). This effect of Ang II is attributed to increases in “nutritive” flow secondary to redistribution of microvascular flow supplying skeletal muscle cells (Rattigan et al., 1996, Newman et al., 2002). Using contrast-enhanced ultrasound technique, Chai et al. have examined the roles of AT<sub>1</sub> and AT<sub>2</sub> receptors on microvascular blood flow in rat skeletal muscle (Chai et al., 2010). Ang II infusions at non-pressor or pressor doses increases hind limb microvascular blood flow. AT<sub>1</sub>R blockade with losartan during systemic Ang II infusion increases hind limb microvascular blood flow and glucose extraction in a NO-dependent fashion suggesting a role for unopposed AT<sub>2</sub>R activation in mediating this effect. Indeed, co-infusion of AT<sub>2</sub>R antagonist, PD123319 significantly inhibited Ang II-induced microvascular dilation and hind limb glucose uptake. These findings suggest that acute effects of Ang II on glucose disposal are mediated by endothelial activation of AT<sub>2</sub>R and in the normal state, it appears that AT<sub>2</sub>R, and not AT<sub>1</sub> exerts a dominant effect on rat skeletal muscle microvasculature (Chai et al., 2010).

The role of AT<sub>2</sub>R in human skeletal muscle microcirculation remains to be clarified. Local infusion of telmisartan (AT<sub>1</sub>R blocker) or PD 123319 (AT<sub>2</sub>R blocker) results in mild increases in forearm blood flow in humans. Moreover, infusion of either telmisartan or PD 123319 attenuates Ang II-induced vasoconstrictor response (Schinzari, Tesauro, Rovella et al., 2011), suggesting that AT<sub>2</sub>R mediates vasoconstriction. Similarly, blockade of AT<sub>2</sub>R in internal mammary arteries from patients with coronary artery disease reduces the vasoconstrictor effect of Ang II (van de Wal, van der Harst, Wagenaar et al., 2007). These results together suggest that AT<sub>2</sub>R like AT<sub>1</sub>R mediates basal and Ang II evoked vasoconstrictive tone in humans. Interestingly, in the same study, CGP 42112A, an AT<sub>2</sub>R-agonist results in a NO-dependent vasodilation (Schinzari et al., 2011). These divergent vascular responses are thought to be due to cell-specific AT<sub>2</sub>R activation in smooth muscle and endothelial cells that mediates vasoconstriction and vasodilation, respectively. Consequently, increases in interstitial Ang II may augment vascular tone through AT<sub>1</sub>R and AT<sub>2</sub>R, while circulating Ang II may decrease vascular tone through AT<sub>2</sub>R activation (Schinzari et al., 2011). However, this assumption needs further evaluation in human microvasculature.

## 7. Angiotensin II and Pathway-Selective Insulin Resistance in the Endothelium

A key feature of insulin resistance is that it is characterized by specific impairment in PI3K-dependent signaling pathways, whereas other insulin-signaling branches including Ras/MAPK-dependent pathways are unaffected (Jiang, Lin, Clemont et al., 1999, Cusi, Maezono, Osman et al., 2000) (Fig. 3). In the endothelium, hyperinsulinemia will overdrive unaffected MAPK-dependent pathways leading to an imbalance between PI3K- and MAPK-dependent functions of insulin. Chronic Ang II exposure and action has been proposed to play a causal role in vascular insulin resistance (Cooper et al., 2007, Kobayashi et al., 2008, Zhou, Schulman and Rajj, 2009, Manrique, Lastra, Gardner et al., 2009). Chronic activation of AT<sub>1</sub>R leads to endothelial dysfunction by increasing oxidative stress, reduced NO bioavailability, impaired generation of endothelium-derived vasodilators, and increased

release and action of vasoconstrictors. Each of these actions of Ang II can independently impair the vasodilatory actions of insulin in the microvasculature. In addition to these deleterious actions, Ang II directly impairs insulin signaling pathways in the endothelium (Kim, Jang, Martinez-Lemus et al., 2012, Maeno, Li, Park et al., 2012, Andreozzi, Laratta, Sciacqua et al., 2004, Oh, Ha, Lee et al., 2011, Presta, Tassone, Andreozzi et al., 2011).

The insulin signaling pathways contains three critical nodes that play an important role in crosstalk with other signaling pathways (Fig. 3). These nodes include, the insulin receptor and its substrates (IRS 1–4), PI3K with its several regulatory and catalytic subunits, and the three AKT isoforms (Taniguchi et al., 2006). Ang II impairs the activity and interaction of these critical nodes in the endothelium. Treatment of bovine aortic endothelial cells with Ang II decreases the number of insulin receptors (IR) in the membrane (Oh et al., 2011). Although, the intracellular mechanisms mediating the reduction in IR are yet to be elucidated, this effect was reversed by ATR blocker. One of the molecular mechanisms for impaired insulin action is increased phosphorylation of IRS-1 at inhibitory serine (Ser) residues (Taniguchi et al., 2006). Phosphorylation of Ser residues appears to interfere with the functional domains of IRS-1. In human umbilical venous endothelial cells (HUVECs), Ang II increases phosphorylation at Ser<sup>312</sup> and Ser<sup>616</sup> of IRS-1 by JNK and ERK1/2, respectively. This increase in inhibitory Ser phosphorylation was associated with impaired interaction with the p85 regulatory subunit of PI3K and consequently reduced activation of Akt and eNOS (Andreozzi et al., 2004). This inhibitory action of Ang II was mediated by the activation of AT<sub>1</sub>R, while inhibition of AT<sub>2</sub>R did not modulate insulin activated IRS/PI3k/Akt/eNOS pathway (Andreozzi et al., 2004, Presta et al., 2011). Similarly, Ang II activates mTOR/p70S6K, leading to phosphorylation of IRS-1 at Ser<sup>636/639</sup> and inhibition of insulin-stimulated phosphorylation of eNOS (Kim et al., 2012). It appears that AT<sub>1</sub>R activation stimulates the mTOR/p70S6K pathway through transactivation of EGFR. Interestingly, NADPH oxidase inhibitors, abrogate Ang II stimulation of mTOR/p70S6K pathway, suggesting a key role for ROS in impaired insulin action in the endothelium. However, in contrast to Ang II-mediated activation of JNK and ERK, which occurred rapidly (<30 min) (Andreozzi et al., 2004, Presta et al., 2011), activation of mTOR/p70S6K pathway required a longer exposure of Ang II (~ 4 hr) (Kim et al., 2012). Moreover, the longer exposure to Ang II was not only associated with increased Ser phosphorylation of IRS-1 but also increased degradation of IRS-1 (presumably ubiquitin-dependent). These findings suggest that Ang II modulates insulin signaling in the endothelium in a time-dependent fashion. Recent study suggests that Ang II also modulates PI3K, another key node for insulin signaling (Maeno et al., 2012). The p85 subunit, a regulatory subunit of PI3K, is critical for interaction with IRS-1 (Taniguchi et al., 2006). In endothelial cells, Ang II causes insulin resistance by activating PKC that subsequently phosphorylates Thr<sup>86</sup> in p85 of PI3K. This inhibitory phosphorylation leads to reduced insulin activation of Akt/eNOS in endothelial cells. Thus, Ang II directly inhibits key signaling molecules in the insulin signaling pathway to selectively impair PI3K/Akt/eNOS pathway resulting in impaired vasodilation and enhanced vasoconstriction that may also lead to impaired insulin-mediated glucose disposal.

## 8. Effects of Angiotensin II on Insulin-Stimulated Skeletal Muscle Microvasculature and Glucose Disposal

Insulin increases skeletal muscle microvascular perfusion to augment glucose disposal. Chronic activation of RAAS has been proposed to play a role in insulin resistance (Cooper et al., 2007, Lastra-Lastra et al., 2009, Manrique et al., 2009). AT<sub>1</sub>R blockade with losartan during concomitant insulin infusion in rats increases insulin-mediated skeletal muscle microvascular perfusion but with no significant effect on glucose disposal (Chai et al., 2011). In contrast, AT<sub>2</sub>R blockade with PD123319, abrogates insulin-induced increases in

capillary recruitment and attenuates glucose disposal (Chai et al., 2011). It appears that PD123319 decreases skeletal muscle uptake by decreasing interstitial insulin levels. These results suggest an important role for AT<sub>2</sub>R in the microvascular actions of insulin. However, the roles of RAS activation and AT receptors in insulin-mediated skeletal muscle capillary recruitment in normal and insulin-resistant states are unknown at this time.

ACE inhibitors and ARB increase insulin mediated glucose disposal in individuals with impaired glucose tolerance, obesity, hypertension or type 2 diabetes mellitus (van der Zijl, Moors, Goossens et al., 2011, Andraws and Brown, 2007, Bosch, Yusuf, Gerstein et al., 2006, Elliott and Meyer, 2007, Paolisso, Balbi, Gambardella et al., 1995, Tocci, Paneni, Palano et al., 2011, Zandbergen, Lamberts, Janssen et al., 2006). In contrast to these observations, acute increases in circulating Ang II levels in healthy individuals augments insulin-mediated glucose disposal (Buchanan, Thawani, Kades et al., 1993, Fliser, Arnold, Kohl et al., 1993, Jamerson, Nesbitt, Amerena et al., 1996, Townsend and DiPette, 1993, Widgren, Urbanavicius, Wikstrand et al., 1993). Thus it appears that the seemingly paradoxical effects of Ang II may be dependent on the duration (acute vs. chronic) and the source of Ang II (interstitial vs. systemic). Whether ACE inhibitors/ARBs affect insulin-induced capillary recruitment in human skeletal muscle is not known. However, chronic administration of quinapril partially reversed impairment in insulin-evoked capillary recruitment and glucose disposal in insulin-resistant Zucker obese rats (Clerk, Vincent, Barrett et al., 2007). Enhanced vasoconstriction is determined by interstitial rather than circulating Ang II (Widdop, Jones, Hannan et al., 2003, Saris, van Dijk, Kroon et al., 2000). Indeed, increasing skeletal muscle Ang II levels (by microdialysis technique) decreases microvascular blood flow in a dose-dependent manner in humans (Goossens, Blaak, Saris et al., 2004). Local RAS activity is increased in insulin resistant states such as obesity, hypertension, and type 2 diabetes mellitus (Luther and Brown, 2011). Consequently, ACE inhibitors or AT<sub>1</sub>R blockers may augment insulin action by decreasing vasoconstrictor tone, reducing oxidative stress, and attenuating insulin resistance. However, in conditions accompanying systemic increases in Ang II such as during chronic treatment with AT<sub>1</sub>R blockers (Levy, 2004) or acute peripheral infusions of Ang II, AT<sub>2</sub>R-mediated vasodilation, as observed in rodents, may play a predominant role in insulin-mediated capillary recruitment and glucose disposal. Clearly these hypotheses need further evaluation in human skeletal muscle microvasculature both in insulin-sensitive and insulin-resistant states.

## 9. Conclusions

Vasodilator actions of insulin are mediated by phosphatidylinositol 3-kinase dependent insulin signaling pathways in endothelium, which stimulate production of nitric oxide. Insulin-stimulated nitric oxide mediates capillary recruitment, vasodilation, increased blood flow, and subsequent augmentation of glucose disposal in skeletal muscle. Pathway-specific impairment of PI3K-dependent insulin signaling pathway leads to metabolic dysfunction and microvascular dysfunction. Similarly, Ang II acts on AT<sub>2</sub> receptor to cause vasodilation and augment insulin-mediated glucose disposal. In contrast, AT<sub>1</sub> receptor activation leads to vasoconstriction, reduced nitric oxide bioavailability, impaired insulin signaling, and insulin resistance. Therapeutic interventions that target the pathway-selective impairment in insulin signaling and the imbalance in AT<sub>1</sub> and AT<sub>2</sub> receptor signaling in microvascular endothelium may simultaneously ameliorate endothelial dysfunction and insulin resistance.

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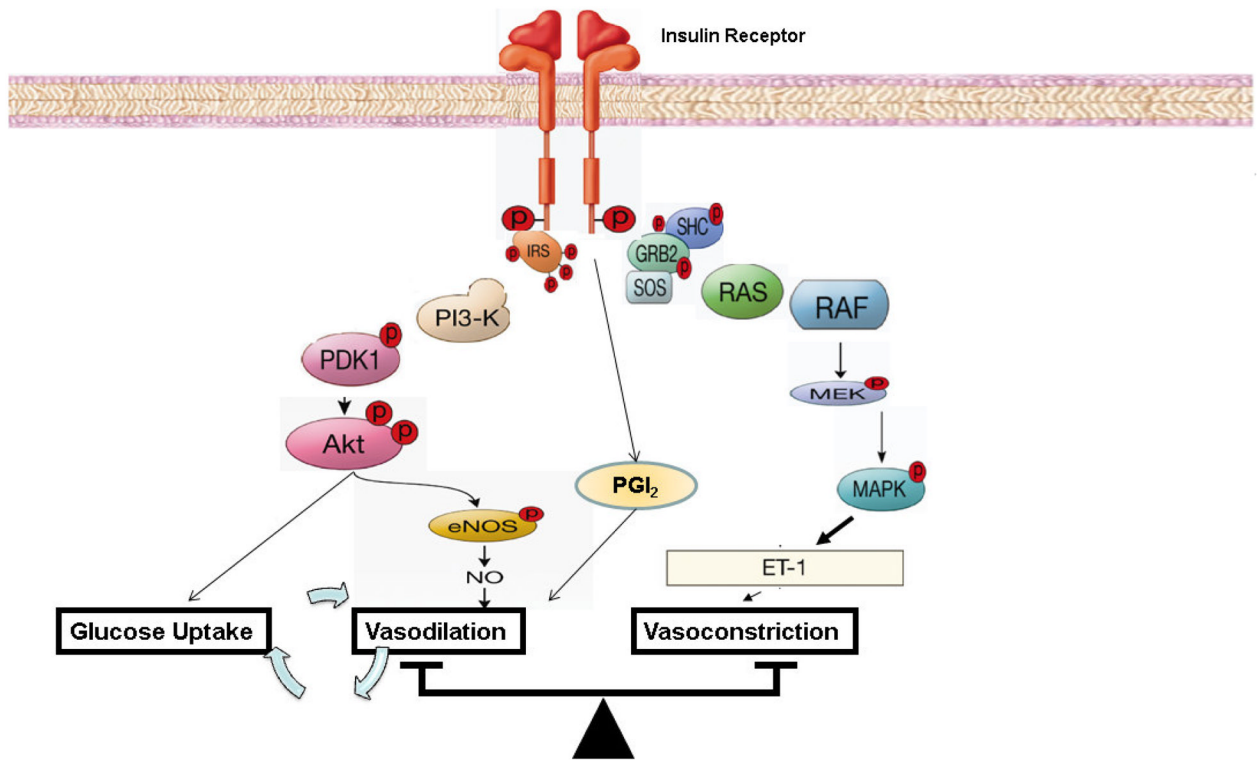
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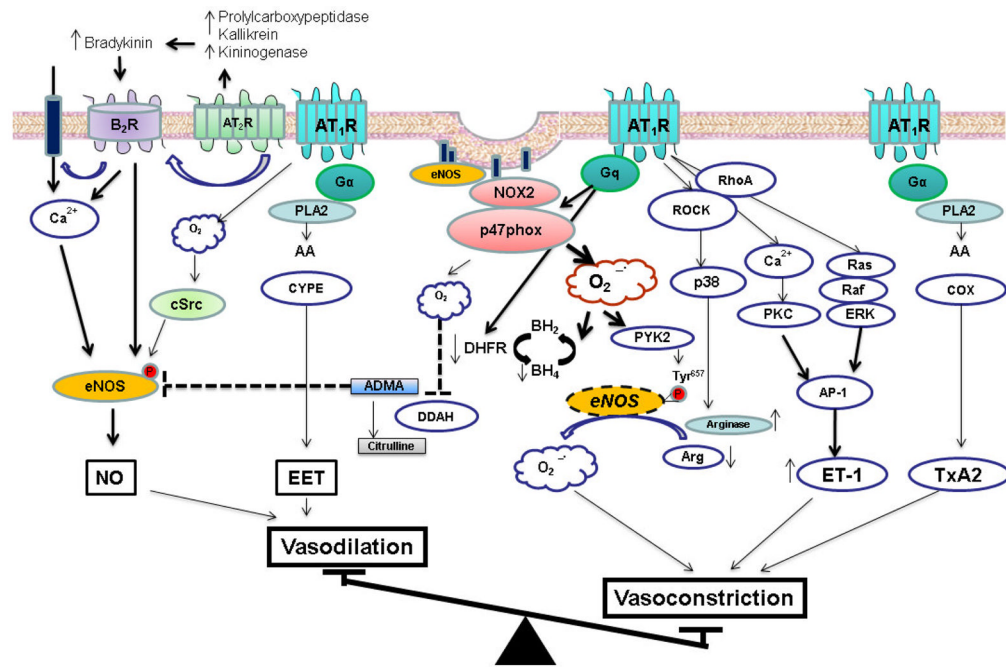
### Highlights

- Vasodilatory actions of insulin in the microvasculature augments glucose disposal.
- Angiotensin II regulates microvascular perfusion and insulin-mediated glucose disposal.
- Chronic activation of angiotensin type 1 receptor impairs vasodilatory actions of insulin.
- Targeting the imbalance in angiotensin II signaling may ameliorate endothelial dysfunction.



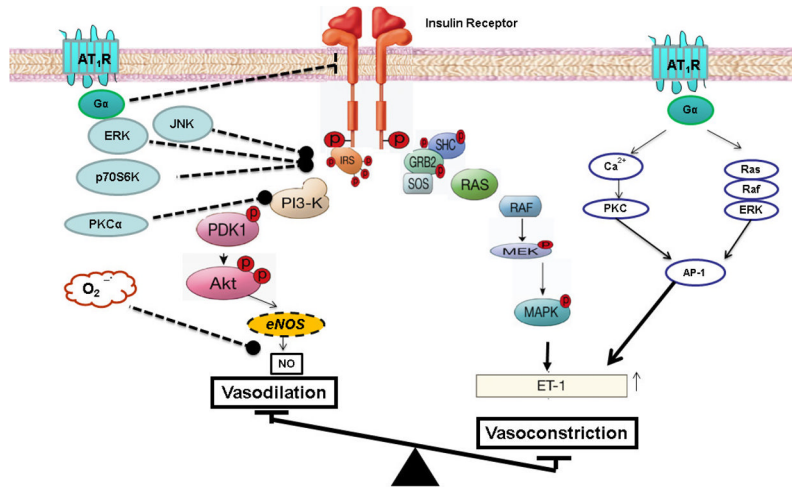
**Figure 1. Insulin signal transduction pathways regulating nitric oxide and endothelin-1 production in endothelium**

PI 3-kinase branch of insulin signaling regulates NO production and vasodilation in vascular endothelium. MAP-kinase branch of insulin signaling controls secretion of endothelin-1 (ET-1) in vascular endothelium. eNOS, endothelial nitric oxide synthase; IRS, insulin receptor substrate; MEK, MAPK kinase; PDK, phosphoinositide-dependent protein kinase.



**Figure 2. Summary of endothelial signaling pathways involved in Angiotensin II modulation of vascular tone**

Angiotensin II activates AT<sub>1</sub>R and AT<sub>2</sub>R in the endothelium. Activation of the AT<sub>2</sub>R increases intracellular acidosis and plasma kallikrein levels which induces the release of bradykinin and binding to the bradykinin receptor (B<sub>2</sub>R). Bradykinin activates endothelial nitric oxide synthase (eNOS) to produce NO. AT<sub>1</sub>R activates NADPH oxidase, a major source for ROS production. ROS in small amounts activates cSrc that subsequently activates eNOS. Chronic AT<sub>1</sub>R activation leads to excessive ROS production that activates PYK2 to inactivate eNOS, reduces BH<sub>4</sub> and L-arginine levels, increases cellular ADMA concentrations, and causes eNOS “uncoupling”. AT<sub>1</sub>R stimulates PKC and Ras/Raf/ERK pathway to increase ET-1 expression. Ang II-induced activation of PLA<sub>2</sub> increases synthesis of vasodilator and vasoconstrictor prostanoids. AT<sub>1</sub>R, angiotensin II type receptor type 1; AT<sub>2</sub>R, angiotensin II type receptor type 2; EET, epoxyeicosatrienoic acid; AA, arachidonic acid; PLA<sub>2</sub>, phospholipase A<sub>2</sub>; PKC, protein kinase C; ROCK, RhoA kinase; ERK, extracellular signal-regulated kinase; PYK2; proline-rich tyrosine kinase 2; p38, p38 mitogen activated protein kinase; COX, cyclooxygenase; AP-1, activator protein-1; TxA<sub>2</sub>, thromboxane A<sub>2</sub>; NO, nitric oxide; ET-1, endothelin-1; BH<sub>4</sub>, tetrahydrobiopterin; BH<sub>2</sub>, dihydrobiopterin; ADMA, asymmetric dimethylarginine; DDAH, dimethylarginine dimethylaminohydrolase; DHFR, dihydrofolate reductase; and CYP2C8, cytochrome P450 epoxygenase.



**Figure 3. Signaling pathways mediating angiotensin II-induced pathway selective insulin resistance in PI3K signaling**

Stimulation of AT<sub>1</sub>R activates PKC, ERK, JNK, and p70S6K to inhibit IRS/PI3K/Akt/eNOS pathway. Increased oxidative stress “uncouples” eNOS and reduces NO bioavailability. Chronic activation of AT<sub>1</sub>R decreases NO production, increases endothelial ET-1 expression, and creates an imbalance between vasodilator and vasoconstrictor actions of insulin. AT<sub>1</sub>R, angiotensin II type receptor type 1; PKC, protein kinase C; IRS, insulin receptor substrate; ERK, extracellular signal-regulated kinase; JNK, C-Jun N-terminal kinase; p70S6K, p70 ribosomal S6 kinase; AP-1, activator protein-1; NO, nitric oxide; and ET-1, endothelin-1.