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The role of GRK2 in hypertension and regulation of GPR30

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A thesis submitted in partial fulfillment of the requirements for the degree in Master of Science

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by

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ABSTRACT

In the hypertensive state, the expression of G protein-coupled receptor kinase 2 (GRK2) level is elevated. On the other hand, the expression of GPR30, a recently discovered GPCR is greatly impaired. The current study focuses on investigating the roles of these two proteins in regulating G protein signaling under the normal and hypertensive states. Angiotensin II and vasopressin were used to examine the effects of GRK2 on G_q coupled GPCR signaling. ERK phosphorylation was proportionally enhanced with GRK2 over-expression. On the other hand, using arborization and wrinkle assays, I have shown that GRK2 acts as a negative regulator of G_s signaling in VSMCs. Aortic ring segments were used to examine the vascular reactivity mediated by GPR30. In WKY rats, the GPR30 agonists aldostrone and G1 attenuated phenylephrine mediated vasoconstriction, while the GPR30 antagonist G15 was able to block the effects of aldosterone but not G1. A wound assay was utilized to estimate the effects of GPR30 activation on endothelial cell migration and proliferation. The G1 effect on wound healing was also seen to be GPR30 independent and EC specific. Overall, these investigations suggest that altering GRK2 expression is able to regulate both G_q and G_s signaling in VSMCs. GPR30 plays a crucial role in vascular reactivity and growth regulatory mechanisms. However, GRK2 and GPR30 do not seem to co-localize or interact in the cell.

Keywords: GRK2, hypertension, GPR30, vascular, G protein signaling

CO-AUTHORSHIP

All adenovirus constructs used were created in our laboratory by Dr. Qingming Ding. All the experiments were carried out in the Gros Laboratory in the Department of Physiology and Pharmacology at The University of Western Ontario. I performed all the experiments described. Dr. Gros provided supervision and funding for all the studies presented in this thesis.

DEDICATION

This Thesis is dedicated to my family

Hua Liu, Yuhua Bai, and Peishen Zhao

For always encouraging me and supporting me to become a scientist

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Finally I would like to dedicate this thesis to my parents and my wife, Peishen, for their unwavering love and encouragement. I would also like to thank all my friends here in Canada and in China for all their thoughtfulness and awesome memories.

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LIST OF ABBREVIATIONS

Abbreviation	Full Name
Aldo	aldosterone
AUC	area under the curve
BCA	bicinchoninic acid
CaM	calmodulin
DAG	diacylglycerol
DMEM	Dulbecco's modified Eagle's medium
E2	17β-estradiol
EC	endothelial cell
ECL	enhanced chemiluminescence
ER	estrogen receptor
ERK	extracellular signal-regulated kinase
EST	estradiol
FBS	fetal bovine serum
FSK	forskolin
GFP	green fluorescent protein
GPCR	G protein-coupled receptor
GPR30	G protein-coupled receptor 30
GRK	G protein-coupled receptor kinase
HEK293	human embryonic kidney 293 cell

IP_3	inositol 1,4,5-trisphosphate
ISO	isoproterenol
KPSS	Krebs physiological salt solution
MLC	myosin light chain
MLCK	myosin light chain kinase
MLCP	myosin light chain phosphatase
MR	mineralocorticoid receptor
PDGF	platelet-derived growth factor
PVDF	polyvinylidene fluoride
PE	phenylephrine
PKA	protein kinase A
PKC	protein kinase C
PLC	phospholipase C
SHR	spontaneously hypertensive rat
SiRNA	small interfering RNA
TBS-T	Tris-Buffered saline, tween-20
VSMC	vascular smooth muscle cell
WKY	Wistar-Kyoto rat

CHAPTER 1

INTRODUCTION

1 INTRODUCTION

1.1 Rationale of the study

Hypertension or elevated arterial blood pressure affects 20% of the adult population in Western industrialized countries and is a major risk factor for myocardial infarction, renal failure and stroke (Lifton, 1996). Many neural, hormonal and cellular abnormalities have been described in the hypertensive state and therefore play an important role in the genesis as well as the maintenance of hypertension. However, the basic abnormality of hypertension appears to be increased peripheral resistance, which reflects a combination of both structural and functional changes to the vasculature (Folkow, 1982). Many different neural, endocrine, paracrine and autocrine factors interact with the vasculature to regulate tone and hence peripheral resistance. This regulation of vascular tone is in part mediated by the activation/inactivation of G protein-coupled receptors (GPCRs). In hypertension, alterations in GPCR activity/function have been described and appear to be related to alterations in GPCR kinase (GRK) activity/expression (Feldman & Gros, 1998, Feldman & Gros, 2006). Indeed, increased GRK2 expression has been observed in both human and animal models of hypertension (Gros et al, 1997, Gros et al, 2000). In addition, more recently the potential role of steroids (aldosterone and estrogen) regulating vascular function and cardiovascular disease have been of keen interest due to the discovery of a GPCR that can mediate the rapid effects of these steroids, namely GPR30. Indeed this GPR30 appears to mediate the rapid vascular effects of

aldosterone and estrogen (Gros et al, 2011a) and may therefore play an important role in the pathogenesis of hypertension, since both alterations in aldosterone and estrogen signaling have been implicated in cardiovascular disease (Feldman & Gros, 2011). Therefore, a better understanding of the acute regulation of vascular GRK2 and the potential interaction with the newly discovered GPCR steroid receptor (GPR30) with GRK2 are important. Thus, the focus of my thesis was: 1) to examine the effects of altering GRK2 expression levels in smooth muscle cells on GPCR signal transduction under normotensive and hypertensive conditions and 2) to examine whether GPR30 function is regulated by GRK2.

1.2 G-protein-coupled receptor kinases and hypertension

1.2.1 Hypertension

Hypertension is a risk factor for myocardial infarction, stroke, and ischemic heart disease. It has been among the top risk factors for death in North America, with an estimated 95% of the Canadian population predicted to be diagnosed with the disease at some point in their life. It often has a multifactorial onset relating to the interplay between environmental and genetic factors, including dietary habits, diabetes, and familial hypercholesterolemia (Harris et al, 2008). Due to its world-wide prevalence, it has become a subject of intensive research, especially because of the many mechanisms through which blood pressure is regulated. Neural and hormonal inputs play the largest roles in blood pressure regulation by maintaining a physiologic blood pressure of 120/80 mmHg in normotensive people (Feldman & Gros, 1998).

1.2.2 GPCR signaling in the vascular system

In vascular smooth muscle cells (VSMCs), an important mechanism which mediates vasodilation is those GPCRs linked to adenylyl cyclase activation through the G_s-proteins (Maguire & Davenport, 2005). Although some of the beta-adrenergic mediated vasodilation is endothelial-dependent (Gros et al, 1994), the beta-adrenergic receptor represents the prototypical vasodilatory receptor in VSMCs. In endothelial cells, GPCRs linked to vasodilatory responses appear more complex and involve GPCRs linked to G_s (β-adrenergic receptors), G_i (muscarinic receptors) and G_q (endothelin-B receptors) resulting in the release of vasodilatory mediators such as nitric oxide, endothelium derived hyperpolarizing factors or prostacyclin (Maguire & Davenport, 2005). On the vasoconstrictor side, GPCRs such as endothelin, alpha-adrenergic and angiotensin receptors and others are linked to the activation of phospholipase C and/or inhibition of adenylyl cyclase via the activation of G_q-proteins and/or G_i-proteins in VSMCs (Feldman & Gros, 1998, Maguire & Davenport, 2005).

1.2.3 Defects in GPCR-mediated vasodilation in hypertension

Although enhanced activation of GPCRs linked to G_q and/or G_i-proteins mediating vasoconstriction have been reported in animal models of hypertension (Touyz & Schiffrin, 2000), many investigators have focused on the GPCR / G_s-protein /adenylyl cyclase complex to explain this defect in receptor-mediated vasodilation during the hypertensive state. The impairment in GPCR-mediated vasodilation appears to be at the level of the receptor. Since either direct acting vasodilators (e.g.

nitroprusside) or vasodilators acting distal to the receptor (e.g. forskolin or dibutyryl cyclic AMP) were not comparably impaired. The efficiency with which GPCRs interact with their G-proteins is in part dependent on the phosphorylation state of the receptor. GPCR phosphorylation is mediated by several different kinases, including the second-messenger dependent protein kinases such as protein kinase A and protein kinase C and by members of the G-protein receptor kinase family (GRKs) (Ferguson, 2001).

1.2.4 GRK and GPCR

Signaling through a GPCR is under tight regulatory control. If a GPCR is subjected to prolonged or repeated stimulation, the receptor undergoes desensitization or down-regulation, which decreases its ability to activate appropriate G-proteins and initiate intracellular signaling cascades. This uncoupling is regulated in part by GRKs that phosphorylate agonist-bound GPCRs (Hargrave et al, 1993). GRK phosphorylation then increases the affinity of the GPCR for the arrestin class of proteins. These receptors can be recycled back to the membrane following dephosphorylation or targeted for down-regulation and degradation (Krupnick & Benovic, 1998). The binding of arrestin initiates down-regulation and prevents the GPCR from associating with its G-protein, thus reducing the functional activity of classical signaling paradigms for that receptor by up to 80% (Freedman & Lefkowitz, 1996).

1.2.5 GPCR activation and desensitization

As shown in *Figure 1.1*, activation of GPCRs following agonist binding induces a conformational change that promotes the exchange of GDP for GTP on the G_{α} subunit and allows the dissociation of the G_{α} and $G_{\beta\gamma}$ subunits (Neer, 1995). Subsequently these G-protein subunits will interact and regulate the activity of a number of other effector molecules, such as adenylyl cyclase, phospholipase C, ion channels, tyrosine kinases and many others (Hamm, 1998). This conformational change also allows the GPCRs to bind one or more of the GRKs. The binding of GRKs, to the agonist occupied receptor, promotes the phosphorylation and desensitization of the GPCR, which promotes the binding of cytosolic proteins termed β-arrestins to the receptor. This leads to a further uncoupling of GPCRs from their G-proteins (Ferguson, 2001, Kohout & Lefkowitz, 2003).

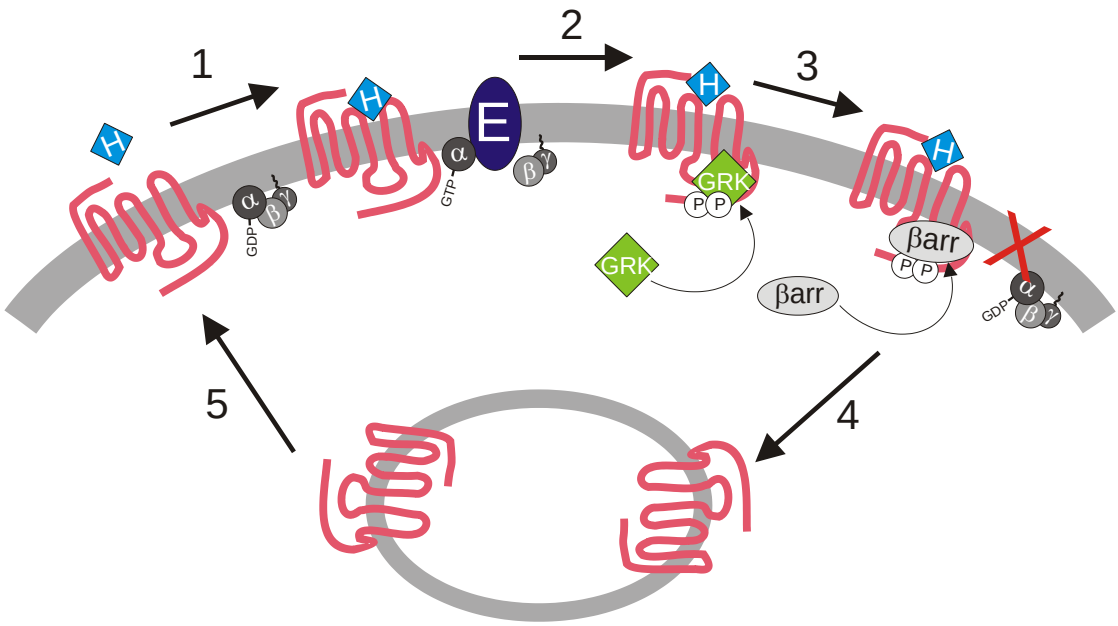

Figure 1.1. Schematic of GPCR activation and desensitization. 1) Hormone (H) binding to GPCR leads to a conformational change of the GPCR, which leads to GDP release from α-subunit of the G-protein and facilitates the binding of GTP. This in turn allows the separation of the G-protein (into α and βγ) subunits which are then able to interact with various effector molecules (E). **2)** Hormone (agonist)-occupied GPCRs also facilitates the binding of GRKs, which in turn phosphorylate and desensitize the GPCRs. **3)** GRK-phosphorylated GPCRs are then further uncoupled from their G-proteins by the binding of β-arrestins (βarr). **4)** Following β-arrestin binding GPCRs are subjected to internalization and de-phosphorylation in endosomes, and **5)** recycled back to the membrane. Figure adapted from references (Feldman & Gros, 1998, Ferguson & Caron, 1998, Metaye et al, 2005).

1.2.6 GRK family

Although there are over 800 known GPCRs in the human genome and with over 100 expressed within the cardiovascular system, it is surprising that only 7 GRKs have been identified (Penn et al, 2000). As shown in *Figure 1.2*, All GRKs have a similar basic structure with an N-terminal domain, a catalytic domain and a C-terminal domain. However, various post-translational modifications are responsible for their regulation within the cell. Based on functional and sequence similarities, the GRK family has been divided into three subfamilies: the rhodopsin kinase subfamily (GRK1 and GRK7), the beta-adrenergic receptor kinase subfamily (GRK2 and GRK3) and the others (GRK4, GRK5 and GRK6) (Premont et al, 1995). GRK1 and GRK7 are found almost exclusively in the retina and modulate opsins. GRK2 and GRK3 are widely expressed, although GRK2 is typically more abundant. GRK4 is found mostly in the testis and proximal tubule of the kidney. GRK5 and GRK6 are widely distributed among tissues. Therefore most GPCRs in the body are regulated by four GRKs: GRK2, GRK3, GRK5 and GRK6. Five of the seven GRKs (GRK1, 4, 5, 6 and 7) are located at the membrane and near activated GPCRs, whereas GRK2 and GRK3 are predominately located in the cytosol and undergo translocation and recruitment to the membrane following GPCR activation (Kohout & Lefkowitz, 2003, Penela et al, 2003). GRK2 has been detected in VSMCs at the protein level (Ishizaka et al, 1997).

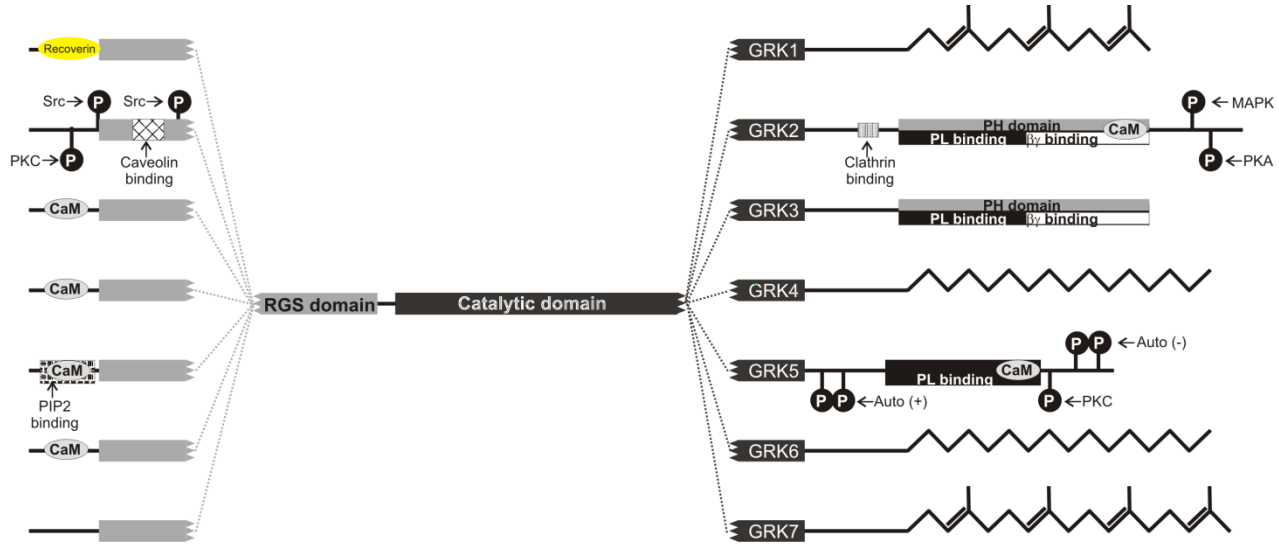

Figure 1.2. Schematic representation of GRK1-7 domain and regulatory sites. GRKs are multi-domain proteins which interact with, and are regulated by, many other cellular proteins. The figure depicts the conserved RGS (regulators of G-protein signaling) domain and the central catalytic domain, common to all seven GRKs. Also depicted are the different functional domains for phosphorylation and interactions with other cellular proteins. CaM, calcium calmodulin regulatory site; PH domain, pleckstrin homology binding domain; PL/PIP2 binding, phospholipids binding domain; βγ binding, G-protein βγ binding domain. GRK5 contains stimulatory (+) and inhibitory (-) auto-phosphorylation sites. Figure adapted from references (Ferguson, 2001, Penela et al, 2003).

1.2.7 The role of GRK2 in vascular system

GRK2 phosphorylates a range of GPCRs (Penn et al, 2000, Pitcher et al, 1998), some of which mediate vasodilation (via G_s-linked receptors) and some mediate vasoconstriction (via G_i and G_q-linked receptors). Therefore, in order for the increase in GRK2 expression/activity to have a role in the development and/or maintenance of hypertension, the increase of GRK2 in hypertensive state would be expected to predominately affect those receptors which mediate vasodilation without altering the activity of receptors mediating vasoconstriction. It is notable that such differential regulation of G_s-linked GPCRs but not G_q or G_i-linked receptors has been demonstrated in FRTL-5 cells over-expressing GRK2 (Iacovelli et al, 1999). However, inhibition of G_q-mediated signaling has been demonstrated in vascular smooth muscle cells over-expressing GRK2 (Peppel et al, 2000). Moreover, in transgenic mice with vascular smooth muscle-specific GRK2 over-expression, angiotensin II-mediated (G_q and/or G_i-linked) increases in blood pressure were almost completely attenuated (Eckhart et al, 2002). This transgenic model demonstrated elevated blood pressures. The net effect may depend on the expression level, the cellular regulation of GRK2, or the model of hypertension studied. In addition to the role of GRK2 in regulating the function of VSMCs, we propose to examine the role of GRKs in endothelial cells. GRK2 is expressed in endothelial cells and may act as the primary regulator of endothelial GPCR function (Tiruppathi et al, 2000, Vinge et al, 2001). In support of this hypothesis, a recent study demonstrated the crucial role of GRK2 in the regulation of sinusoidal endothelial cell nitric oxide synthase function in rats with portal

hypertension (Liu et al, 2005).

1.2.8 GRK and hypertension

GPCRs play a critical role in the regulation of vascular tone. As shown in *Figure 1.3*, the coupling of GPCRs to different subclasses of G-proteins (G_s, G_i and G_q) allows for very diverse intracellular signaling events. Moreover, we and others have shown that GPCR-linked vasodilation is impaired during the hypertensive state. This impairment in GPCR-mediated vasodilation has been characterized as an uncoupling of the receptor from its G-protein (Feldman & Gros, 1998). G-protein-coupled receptor kinases (GRKs) phosphorylate and uncouple GPCRs from G-proteins and their effector systems. Several lines of evidence have now demonstrated increased GRK expression/activity in both human and animal models of hypertension (Eckhart et al, 2002, Felder et al, 2002, Gros et al, 1997, Gros et al, 1999, Gros et al, 2000, Ishizaka et al, 1997, Keys et al, 2005). Taken together, all these studies demonstrate a potential crucial role for GRKs in the pathogenesis and/or maintenance of hypertension. However, GRKs can mediate the phosphorylation of many different GPCRs, including those GPCRs linked to both vasodilation and vasoconstriction (Pitcher et al, 1998). Why increased GRK expression preferentially affects those GPCRs linked to vasodilation during the hypertensive state is still unclear.

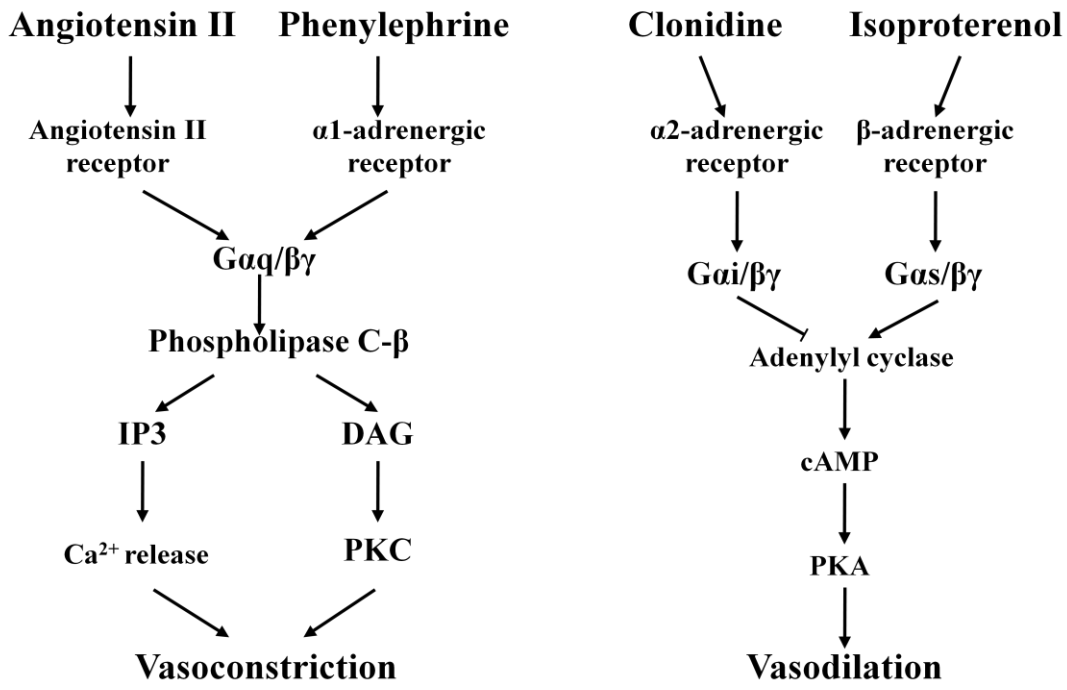

Figure 1.3. GPCR signaling in vascular smooth muscle cells. Activation of $G_{\alpha s}$ stimulates adenylate cyclase (adenylyl cyclase), which leads to the formation of cAMP and subsequent phosphorylation of intracellular targets by PKA (protein kinase A). PKA phosphorylation causes increased relaxation in smooth muscle cells ultimately leading to vasodilation. $G_{\alpha i/o}$, on the other hand, inhibits adenylate cyclase activity, decreasing cAMP formation and PKA phosphorylation, effectively decreasing relaxation and diminishing vasodilation. $G_{\alpha q}$ activates PLC (phospholipase C) leading to the formation of IP_3 (inositol 1,4,5-trisphosphate) and DAG (diacylglycerol). IP_3 initiates Ca^{2+} release from intracellular stores, which activates Ca^{2+}-CaM (calmodulin), leading to contraction. Additionally, DAG activates PKC (protein kinase C), which also phosphorylates target proteins that facilitate smooth muscle contraction leading to vasoconstriction.

1.3 G-protein-coupled Receptor 30 and cardiovascular function

Steroid hormones such as aldosterone and estradiol have been increasingly appreciated as important physiological and pathophysiological regulators of cardiovascular functions. These hormones were conventionally thought to act solely as transcriptional regulators. However, it is now known that they mediate their actions both via "classical" transcriptional mechanisms as well as via "rapid" (previously termed "nongenomic") mechanisms. A recently recognized G protein coupled receptor, GPR30 has been shown to mediate some of the rapid effects of estradiol and aldosterone (*Figure 1.4*).

1.3.1 Cellular location and signaling of GPR30

Although ER_{α} and ER_{β} are accepted as the predominant nuclear receptors involved in the genomic effects of estrogen, evidence also indicates that rapid modulation of cell-signaling pathways occurs via a subpopulation of ERs located at the plasma membrane (Edwards, 2005), which has fueled the speculation about a role of GPR30 (Levin, 2011). The localization of GPR30, however, seems to be predominantly intracellular (Otto et al, 2008), consistent with reports that describe the constitutive internalization of plasma membrane GPR30 (Sanden et al, 2011).

Signaling through GPR30 occurs via transactivation of the epidermal growth factor receptor (EGFR) and involves nonreceptor tyrosine kinases of the Src family (Filardo et al, 2000). In this mechanism, which is now also accepted for other G-protein-coupled receptors (Bhola & Grandis, 2008), stimulation of GPR30 activates

metalloproteinases and induces the release of heparin-binding EGF, which binds and activates EGFR (Prenzel et al, 1999), leading to activation of downstream signaling molecules, such as ERK1/2 (Edwin et al, 2006). Moreover, 17β-estradiol-mediated activation of GPR30 stimulates production of cAMP, intracellular calcium mobilization and PI3K activation (Revankar et al, 2005, Thomas et al, 2005). Further research in human breast cancer cells suggests that sphingosine kinase (Sukocheva et al, 2006) and integrin $\alpha_5\beta_1$ (Quinn et al, 2009) are intermediates in 17β-estradiol-mediated EGFR transactivation; the latter study suggesting a role for GPR30 in fibronectin assembly.

In addition to the above-mentioned rapid signaling events, GPR30 also regulates transcriptional activity, albeit indirectly, by activating signaling mechanisms that involve cAMP, ERK and PI3K (Meyer et al, 2009). The genes regulated by GPR30 include FOS, which encodes c-Fos (Maggiolini et al, 2004), a protein that forms a heterodimer with various other proteins to form the transcription factor AP-1. In turn, these signaling pathways also activate other transcription factors, such as steroidogenic factor 1 (Lin et al, 2009), which induce expression of additional genes (Prossnitz & Maggiolini, 2009).

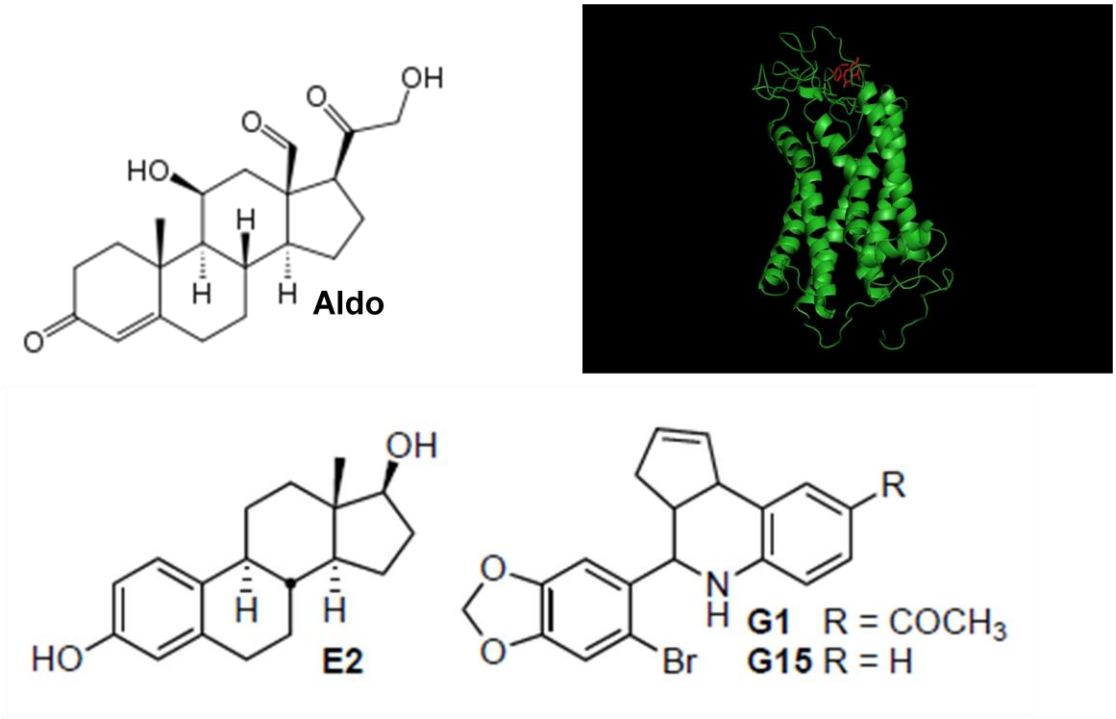

Figure 1.4. Structures of aldosterone (Aldo), 17β-estradiol (E2), GPR30-selective agonist (G1) and antagonist (G15). G1 and G15 have similar structures as that of aldosterone or estradiol. They can bind to the extracellular domains of GPR30.

1.3.2 Steroid hormones mediated rapid vascular and metabolic effects

Clinical trials have suggested that aldosterone and estradiol have effects beyond those mediated via their "classical pathways". For example, in patients with congestive heart failure or following myocardial infarction, the beneficial effects of aldosterone antagonists have been demonstrated (Pitt et al, 1999, Pitt et al, 2003) - not explainable by their weak diuretic effects. These observations have triggered the ongoing study of mechanisms by which aldosterone might mediate the detrimental cardiovascular effects that are alleviated by aldosterone antagonists. Our studies have demonstrated rapid aldosterone actions on vascular reactivity (Liu et al, 2003), single cell contractility (Gros et al, 2007) and growth regulatory mechanisms due to activation of several receptors, including GPR30 (*Figure 1.5*). In regards to estradiol, its potentially beneficial cardiovascular effects have long been appreciated (Miller & Duckles, 2008).

However, the mechanisms underlying the "paradoxical" detrimental cardiovascular effects of postmenopausal estrogen treatment seen in randomized clinical trials (Grady et al, 2002, Rossouw et al, 2002) have suggested that estradiol might mediate both beneficial and detrimental effects. Our recent studies have demonstrated that estradiol could have diametrically opposite effects on vascular smooth muscle function, depending on whether GPR30 or the estrogen receptor (ER) was activated (Ding et al, 2009). In summary, estradiol and aldosterone activation of both "classical" steroid receptors (MR, ER) and GPR30 are important in cardiovascular and metabolic regulation. Delineating which steroids interact with

which receptors in the regulation of which processes would be the first step in developing novel therapeutic approaches to selectively modulate their pathobiological actions.

1.3.3 The role of GPR30 in steroid hormone-mediated vascular effects

Recent studies have indicated that the rapid vascular effects of aldosterone and estrogen are mediated by both their classical receptors (MR and ER, respectively) as well as by GPR30 (Gros et al, 2011a). GPR30 was initially characterized as mediating the rapid effects of estrogen (Revankar et al, 2005). GPR30 is a widely expressed GPCR (expressed in heart, arteries, breast, lung, central nervous system and leukocytes) (Hasbi et al, 2005) and is detectable in freshly isolated vascular smooth muscle cells (Ding et al, 2009) as well as in vascular endothelial cells (Gros et al, 2011b). GPR30 has been studied predominantly in the context of reproductive biology and cancer biology. Initial studies primarily focused on GPR30's growth regulating effects (Ahola et al, 2002, Albanito et al, 2007, Filardo, 2002, Vivacqua et al, 2006b), the impact of its expression on cancer survival (Filardo et al, 2006, Smith et al, 2007, Vivacqua et al, 2006a) and GPR30's actions on oocyte maturation (Kolkova et al, 2010, Pang & Thomas, 2010, Wang et al, 2007).

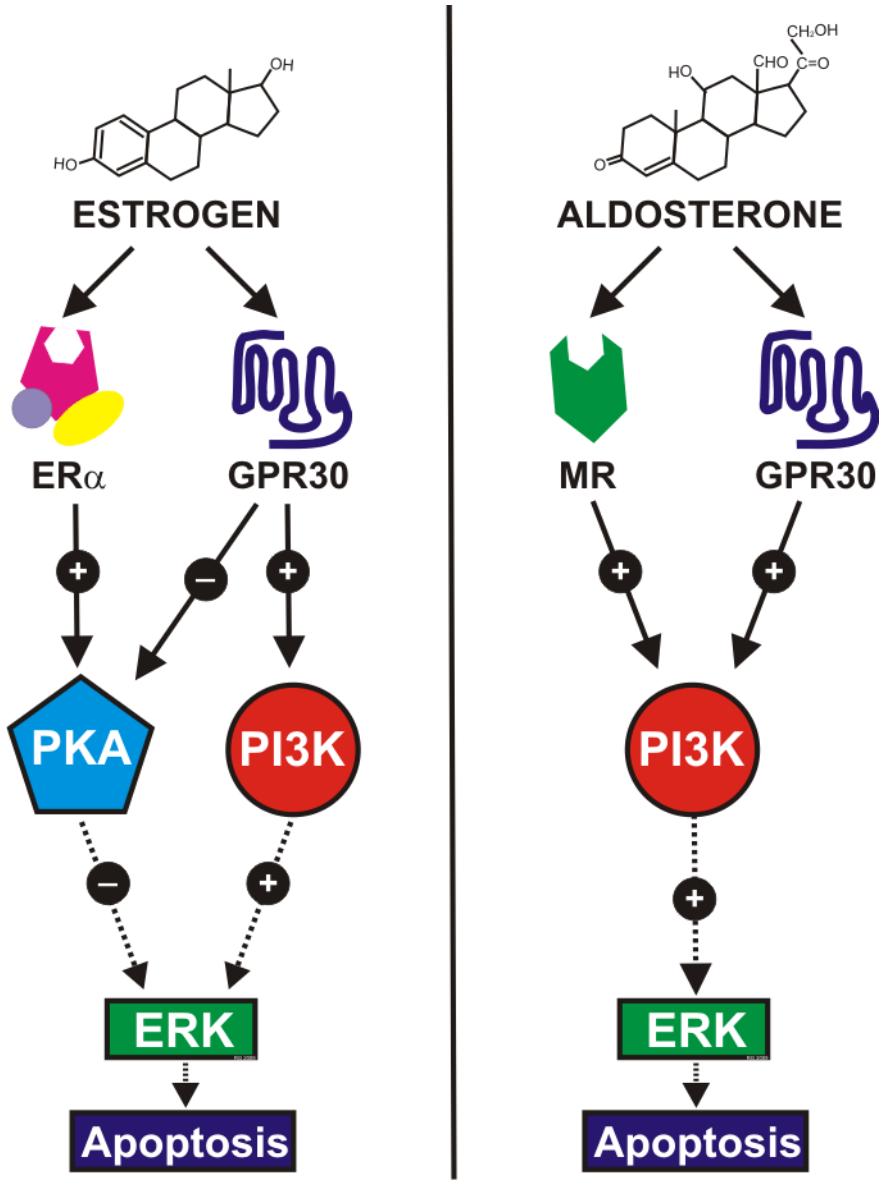

Figure 1.5. **Schematic depiction of divergent pathway activation by estrogen and aldosterone acting through ERα/MR and/or GPR30-linked pathways resulting in the activation or inhibition of ERK activity/ERK-dependent apoptosis.** PI3K: Phosphoinositide-3 kinase; PKA: Protein kinase A. Figure adapted from reference (Feldman & Gros, 2011)

1.3.4 GPR30 and regulation of vascular reactivity

Studies of the cardiovascular effects of GPR30 have mostly focused on its effects on vascular reactivity. GPR30 activation lowers blood pressure both acutely (Haas et al, 2009) and chronically (Lindsey et al, 2009). Genetic deletion of GPR30 has been associated with sex-specific increases in blood pressure (Martensson et al, 2009), although we are aware that the generalizability of this finding in other GPR30 deletion models has not been universal (Langer et al, 2010). Our studies demonstrated that, in vascular smooth muscle cells, GPR30 activation mediates myosin light chain phosphorylation (Gros et al, 2007, Gros et al, 2011b). However, since the overall effect of vascular GPR30 activation is vasodilatory and GPR30-mediated vasodilation is endothelium-dependent (Broughton et al, 2010), this would suggest that understanding how GPR30 regulates endothelial cell-based mechanisms is critical for understanding how GPR30 regulates vascular reactivity.

1.3.5 The effect of GPR30 on the regulation of vascular growth

As noted above, GPR30 activation has prominent effects in regulating cancer cell growth. GPR30's role in regulating cardiovascular cell growth is unknown, but could be important in settings where vascular cell growth is stimulated- e.g., following vascular injury or occlusion. Recent studies by our laboratory have shown that in vascular smooth muscle cells GPR30 activation is pro-apoptotic and is directly antagonist to the anti-apoptotic consequences of ERα activation. In other cell systems GPR30 activation has been linked to either anti-proliferative/pro-apoptotic (Ariazi et

al, 2010, Chan et al, 2010) or pro-proliferative/anti-apoptotic (He et al, 2009, Lin et al, 2009, Pandey et al, 2009) consequences. The role of GPR30 in regulating endothelial cell growth is unknown. Our *in vitro* observations (Ding et al, 2009) showed that GPR30 expression is down-regulated in the setting where vascular smooth muscle cells undergo the phenotypic switch from a contractile to a synthetic cell type following primary culture (Li et al, 1999). A similar switch to a synthetic phenotype occurs with vascular injury (Clowes et al, 1983). Based on our preliminary studies suggesting that GPR30 is down-regulated in vascular smooth muscle cells following injury, we propose that reversal of this down-regulation of GPR30 response might have a significant effect on the smooth muscle response to injury. Together, these studies delineated the effect of GPR30 on the regulation of vascular growth.

1.4 Research objectives

Based on the data presented in the introduction of this thesis, I hypothesized the following:

1) The extent of GRK2 content is essential for the development and maintenance of the vascular hypertensive phenotype.
2) GPR30 plays a crucial role in vascular reactivity and growth regulatory mechanisms.
3) GRK2 mediates the desensitization of GPR30 and regulates GPR30 signaling.

1.5 References

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CHAPTER 2

MATERIALS AND METHODS

2 MATERIALS AND METHODS

2.1 Animal protocols

Male Wistar-Kyoto rats (WKY) and spontaneously hypertensive rats (SHR) (10-12 weeks of age; Charles River, Pointe-Claire, QC) were utilized in our studies. They were cared for in accordance with the Canadian Council on Animal Care guidelines and housed under a 12 hours light/dark cycle with free access to standard laboratory chow and drinking water. The protocol for their use was approved by the Animal Use Subcommittee (AUS) of the University of Western Ontario. Systolic blood pressure was determined via tail-cuff plethysmography as previously described (Gros et al, 2000). Mean systolic pressures in SHR were significantly higher compared with WKY rats.

2.2 Rat aortic vascular smooth muscle cell primary cultures

Rat aortic vascular smooth muscle cell (VSMC) primary cultures were isolated by a modification of the methods of Touyz et al. (Touyz et al, 1994). Briefly, freshly isolated aortas from both normotensive and hypertensive rats were concurrently digested using collagenase and elastase incubations as we have previously described (Gros et al, 2006b). Rats were anaesthetized, and the chests were opened. The aortas were located, dissected out, and placed in ice-cold PBS. Blood and connective tissue were removed, and the aortas were cut into longitudinal strips. Aortic tissue strips from 2 aortas were placed in a sterile 50 ml centrifuge tube (Falcon, VWR Canlab,

Mississauga, ON) containing 1 mg/ml collagenase (Worthington Biochemical Corporation, Lakewood, N.J.) in 5 ml of Ham's F12 medium (Gibco BRL, Burlington, ON) supplemented with 1% gentamicin and 10% FBS, and incubated at 37℃ for 20 minutes. The medium was aspirated and replaced with 5 ml of fresh medium containing 1 mg/ml collagenase, and incubated at 37℃ for 30 minutes. An additional 5 ml of medium containing 1 mg/ml collagenase was added to the tube and incubated for another 30 minutes. The tube was then centrifuged for 5 minutes at 2000 rpm at room temperature and the supernatant was aspirated. The tissue pellet was resuspended in 5 ml of medium containing 1 mg/ml collagenase and 0.5 mg/ml elastase (Worthington Biochemical Corporation, Lakewood, N.J.), and incubated at 37℃, shaking periodically, for 90 minutes. The solution was collected with a pipette and filtered through a sterile 100 μm cell strainer (Nitex, VWR Canlab, Mississauga, ON) into another sterile 50 ml conical tube. The filter was washed with 5 ml Dulbecco's modified Eagle's medium (DMEM) (Gibco BRL, Burlington, ON). The filtered solution was centrifuged for 5 minutes at 2000 rpm at room temperature and the supernatant was aspirated. Finally, the pellet was resuspended in DMEM supplemented with 10% FBS, 1% fungizone and 0.1% gentamicin, and the cells were plated onto 100 mm culture dishes. Cells were maintained in a Hera Cell incubator (TC Tech Products, Maple Plain, MN) at 37℃ and 5% CO_2. VSMCs were used between passages 4 and 12 for all experiments. Both early and late passages of cells were used for all assays to negate the possibility of differential effects reflected because of passage-specific differences in VSMCs.

2.3 Rat aortic endothelial cell cultures

Rat aortic endothelial cells (ECs) were purchased from Cell applications Inc. (San Diego, CA) and cultured according to the instructions of the supplier as we have recently described (Gros et al, 2011b). Growth medium, subculture reagent kit and attachment factor solutions were also purchased from Cell applications Inc. Culture dishes were precoated with Attachment Factor Solution for 30 minutes at 37℃ or 2 hours at room temperature. When subculturing ECs, monolayer of cells were washed with HBSS, and 5 ml of Trypsin/EDTA Solution was pipetted into the culture dish. After gently rocking the dish, 3 ml of the solution was removed immediately. When most of the cells were detached, 5 ml of Trypsin Neutralizing Solution was added to the dish to inhibit further tryptic activity. The cell suspension was then transferred to a 14 ml sterile conical tube and centrifuged at 1000 rpm for 5 minutes. The supernatant was aspirated from the tube without disturbing the cell pellet. Finally, cells were resuspended in 10 ml of Rat Endothelial Cell Growth Medium and inoculated into Attachment Factor Solution coated dishes. ECs were utilized between passages 4 and 10.

2.4 GRK2 protein expression in vascular smooth muscle cells

Assessment of GRK2 protein expression was determined by immunoblotting as previously described (Gros et al, 2000). Briefly, cultured VSMCs from WKY and SHR rats were washed with PBS and were lysed with ice-cold lysis buffer (40 mM Tris-HCl, pH 7.4, 274 mM NaCl, 2% Nonidet P-40 and 1 EDTA-free protease

inhibitor cocktail tablet (Roche Diagnostics, Indianapolis, IN)). Cells were scraped off the dishes, placed into labeled eppendorf tubes, and centrifuged at 13 000 rpm for 10 minutes at 4 ℃. The protein concentration of the supernatant within the samples was then measured using a bicinchoninic acid (BCA) assay (Thermo Scientific, Rockford, IL). In this assay, 10 µl of the sample supernatant was added to 3 ml of the BCA reagents and the solutions were incubated for 30 minutes at 37 ℃. The absorbance of the samples was then read at 562 nm using a Spectronic 20 spectrophotometer (Bausch & Lomb, NY). Equal protein concentrations of the samples were then run on a 10% SDS-PAGE gel in addition to a Precision Plus Protein Dual Color Standards Marker (Bio-Rad, Hercules, CA). The gels were blotted electrophoretically onto an Immuno-Blot polyvinylidene fluoride membrane (Bio-Rad, Hercules, CA). The membranes were then blocked with 5% blocking buffer (Tris-Buffered saline, tween-20 (TBS-T), powdered Skim Milk) and incubated at 4 ℃ overnight in 1:1000 GRK2 antibody (Santa Cruz Biotechnology, Santa Cruz, CA), followed by a 30 minutes wash with TBS-T, then 1:5000 anti-rabbit antibody (Sigma-Aldrich, Oakville, ON) for 1 hour at room temperature. The blots were then washed again for 30 minutes with TBS-T. GRK2 protein bands were then detected and visualized by enhanced chemiluminescence according to the protocol described by the assay manufacturer (PerkinElmer Life and Analytical Sciences, Waltham, MA). Western blots were then analyzed using Scion Imaging for Windows software (Frederick, MD), which determined the intensity of the bands of proteins of interest.

2.5 Adenoviral constructs

Adenoviral constructs were generated with AdMax^{TM} adenovirus vector creation kit as per manufacturer's instructions (Microbix BioSystems Inc., Toronto, ON) as we previously described (Gros et al, 2011b). Briefly, GFP, GRK2 or GPR30 cDNA were generated by PCR using plasmid templates of GFP cDNA (Clontech, Mountain View, CA), GRK2 or GPR30 (ATCC), the resultant cDNAs were subcloned into shuttle vector pDC316 and purified. For adeno siRNA constructs, the small interfering RNA (siRNA) sequences specific for GFP (bp 418-438), GRK2 (bp 710-730) and GPR30 (bp 356-376) were cloned into the modified pDC312 vector. The recombinant plasmid was then co-transfected into human embryonic kidney (HEK) 293 cells with adenoviral DNA pBHGlox (delta) E1, 3Cre. Recombinant adenovirus was harvested by lysis of transfected HEK293 cells using 3 freeze/thaw cycles. For gene transfer, VSMCs or ECs were infected with adenoviral constructs for 24 hours at 37 ℃, following which, medium was disposed of and replenished. Cells were utilized for experimentation 48 hours after gene transfer.

2.6 GRK2 over-expression & knockdown by adenoviral infection

WKY and SHR VSMCs were infected with increasing concentrations of GRK2 or SiGRK2 adenovirus, GFP or SiGFP virus was used as control. The media was changed once after 24 hours. 48 hours post-infection, VSMCs were washed with PBS and lysed with ice-cold lysis buffer (40 mM Tris-HCl, pH 7.4, 274 mM NaCl, 2% Nonidet P-40 and 1 EDTA-free protease inhibitor cocktail tablet (Roche Diagnostics,

Indianapolis, IN)). Western blots were used to determine the virus concentration needed to increase or decrease GRK2 protein expression levels by approximately 50%.

2.7 Immunoblotting for ERK phosohorylation

To examine the rapid effects of "vasoconstrictor" hormones acting through GPCRs linked to G_q signaling, I examined the short-term effects of angiotensin II and vasopressin. The effects of these hormones on ERK activation were compared with that of platelet-derived growth factor (PDGF, hormone acting through a tyrosine kinase receptor pathway, as opposed to a GPCR pathway). As previously described (Gros et al, 2006a), VSMCs were infected with GRK2 or SiGRK2 adenovirus for 24 hours. After serum starvation for 24 hours, cells were incubated in the presence or absence of 1 μM angiotensin II, 1 μM vasopressin, or 20 ng/ml PDGF at 37℃ for 5 minutes. VSMCs were then washed twice with ice-cold PBS and directly lysed in a buffer containing 10 mM Tris-HCl, pH 7.4, 10 mM NaCl, 3 mM $MgCl_2$, 0.3% Nonidet P-40, and 1 mM Na_3VO_4 with 1 mM phenylmethylsulfonyl fluoride. The cell lysates were centrifuged at 13000 rpm for 10 minutes at 4℃. 50 μg of protein was resolved on 10% SDS-PAGE and blotted electrophoretically onto Immun-Blot polyvinylidene fluoride membrane (Bio-Rad, Hercules, CA). The membranes were blocked with 5% skim milk and incubated with either 1:1000 anti-phospho-ERK1/2 or 1:1000 anti-ERK1/2 (BD Transduction Laboratories, Lexington, KY), followed by 1:5000 anti-rabbit antibody (Sigma-Aldrich, Oakville, ON). Bands were visualized by

chemiluminescence as described by the manufacturer's protocol (PerkinElmer Life and Analytical Sciences, Boston, MA). Total-ERK1/2 was used as loading control.

2.8 Arborization of VSMCs in response to drug treatment

Short-term β-adrenergic effects on contractile function were determined by assessment of the extent of vascular smooth muscle arborization (Nabika et al, 1985, Nabika et al, 1988). The arborization response mediated by elevation of cAMP has been linked to cytoskeletal changes, including reorganization of actin fibers (Ben-Ze'ev & Amsterdam, 1987, Westermark & Portor, 1982) and assembly of microtubules (Nabika et al, 1985). As previously described (Gros et al, 2006a), VSMCs were cultured onto 35 mm dishes. Cells were infected with GFP, SiGFP, GRK2, or SiGRK2 adenovirus for 24 hours before being serum-starved for another 24 hours using starvation medium (DMEM supplemented with 0.1% FBS, 50 units/ml gentamicin, and 1% Fungizone). Following serum starvation, the medium was then replaced by M199 medium and the cells were returned to the incubator for 15 minutes and then transferred to a temperature controlled chamber for another 15 minutes maintained at 37℃ (Bionomic controller; 20/20 Technology Inc., Wilmington, NC) on an inverted microscope (Axiovert S100; Carl Zeiss, Thornwood, NY). Cells were then treated with either isoproterenol (ISO, 10 μM) or forskolin (FSK, 10 μM) obtained by Sigma-Aldrich (Oakville, ON). The progression of the arborization response was measured in the dark using time-lapse video microscopy. One picture was taken prior to treatment to show the initial morphology of the cells and this

baseline served as a control. The other picture was obtained after 40 minutes and the extent of arborization was assessed by determining the change in image intensity using the threshold setting within the image analysis software (Northern Eclipse 6.0; Empix Imaging, Toronto, ON) (*Figure 2.1*). The change in image intensity was expressed as a percentage of basal intensity (before the addition of drug).

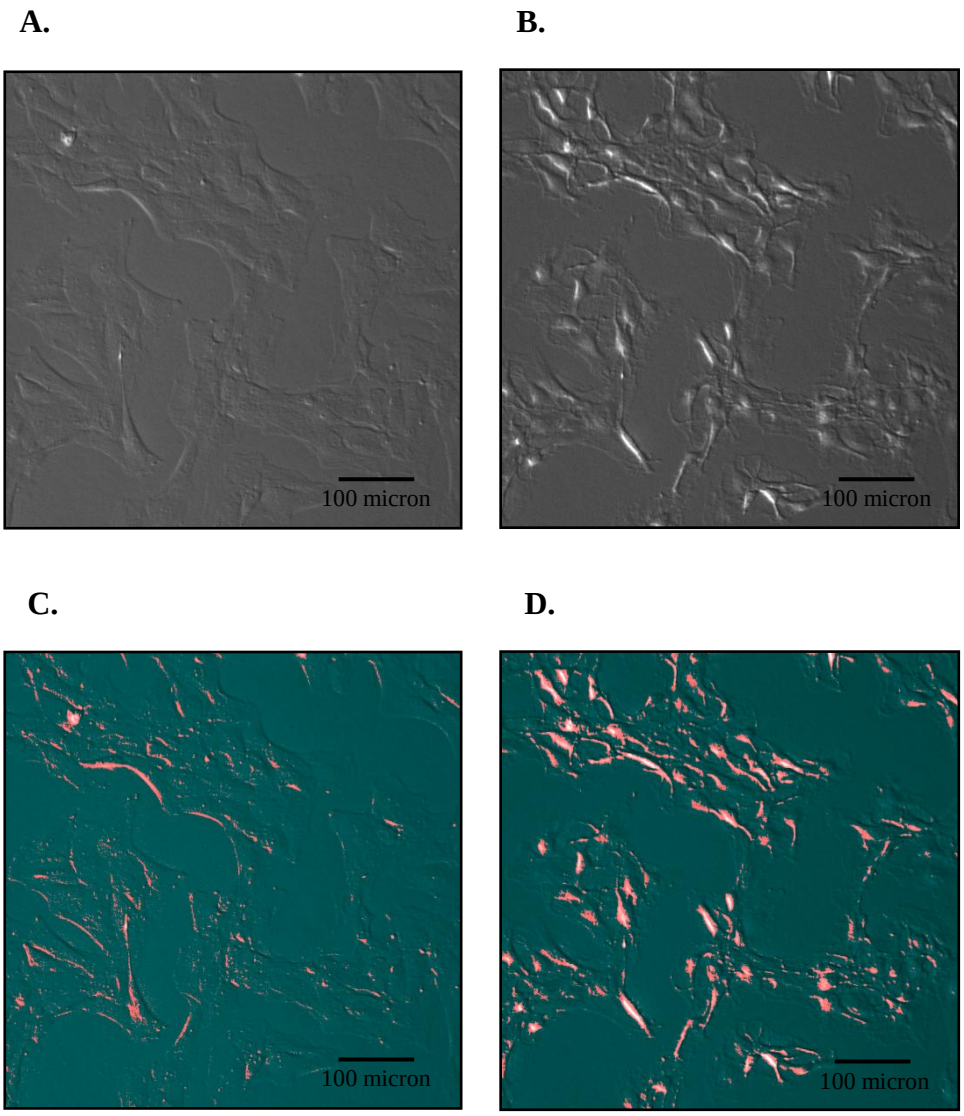

Figure 2.1. Representative images of VSMCs arborization. I utilized the arborization "retraction" process of cultured VSMCs as a measure of functional responses to G_s-mediated GPCR activation. Arborization is a rapid and striking morphological change of vascular smooth muscle cells, characterized by central cell rounding and the presence of long branching processes. It is caused by increased cAMP and has been proposed as an index of vasodilator reactivity. **A.** baseline; **B.** 40 minutes post-10 μM ISO treatment; **C.** baseline upon thresholding; **D.** threshold of 40 minutes post-10 μM ISO treatment.

2.9 Assessment of VSMCs contractility using the silicone wrinkle assay

This assay was modified from the methods of Harris et al. (Harris et al, 1980). As recently described (Gros et al, 2011a), small drops of silicone were placed in the center of glass cover slips and were allowed to flatten by gravity. The glass cover slips were then passed through a Bunsen burner flame to cross-link the top layer of the silicone, to permit subsequent cell attachment, and placed in culture dishes (35 mm diameter). VSMCs were seeded on the siliconized cover slips at a density of 2.5×10^5 cells/ml, and maintained in DMEM supplemented with 10% FBS, gentamicin, and fungizone. The VSMCs attach to the polymerized silicone, resulting in substrate deformation of the weakly cross-linked top silicone layer. The DMEM was replaced with Medium 199 (Gibco) with Hank's salts, L-glutamine, and 25 mmol/L HEPES buffer, without FBS, 18 hours before contractility measurements, and cells were maintained in an incubator until assay. To assess substrate deformation, an indirect measure of VSMCs tone, 35 mm dishes containing the siliconized cover slips were mounted in a temperature-controlled chamber (37 ℃, Bionomic controller BC-100, 20/20 Technology, Inc., Wilmington, NC) on an inverted microscope (Zeiss, Axiovert S100). Cells were allowed to stabilize in this microscope chamber for 15 minutes prior to treatment. After 10 minutes of baseline recording, increasing doses of ISO or FSK (0.1 nM-10 μM) were given to VSMCs at 5 minutes intervals. Contractile responses were assessed using time-lapse video microscopy. Wrinkle area was quantified using bright image thresholding (Northern Eclipse 6.0, Empix Imaging, Toronto, ON), where the relaxation was reflected as decreased intensity (*Figure 2.2*). Contractile responses were expressed relative to baseline intensity measured before treatment.

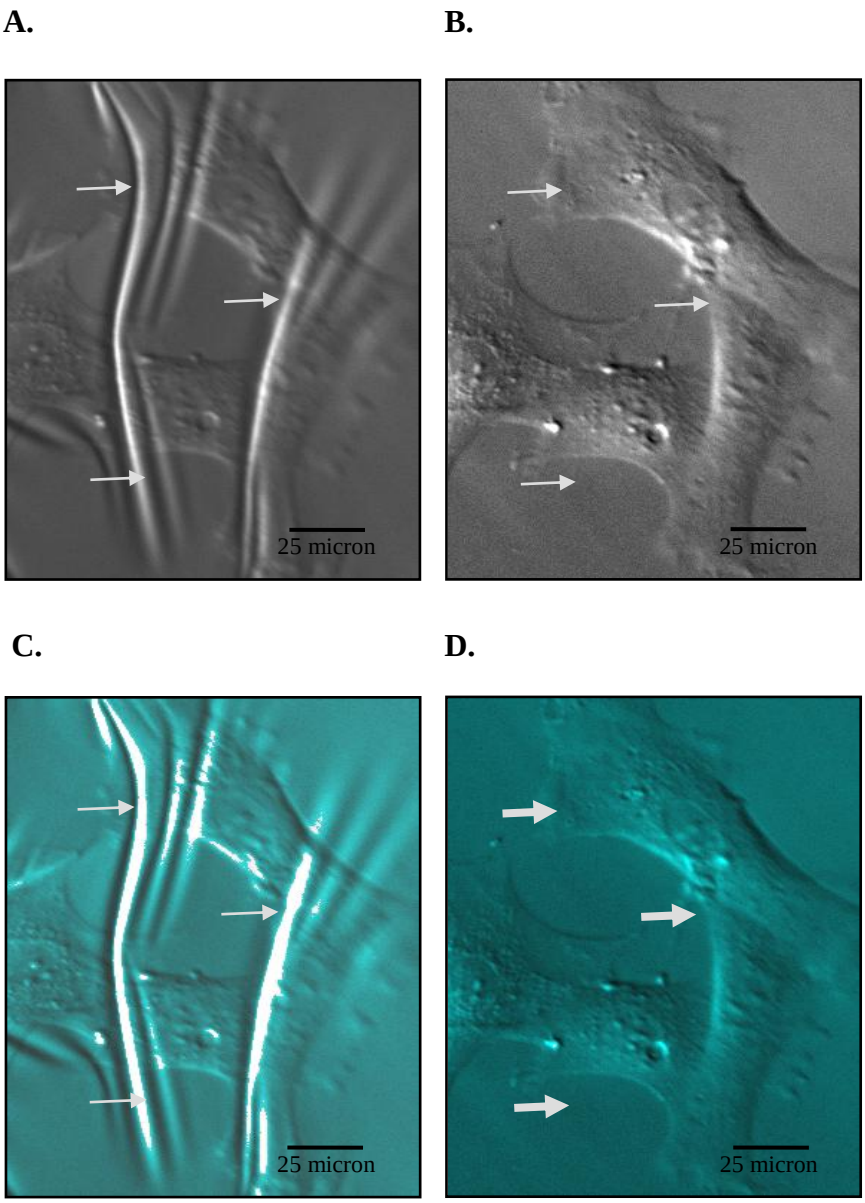

Figure 2.2. Representative microscopic samples (magnification 400X) illustrating wrinkles and thresholding of VSMCs using the silicone wrinkle assay. The intensity of the wrinkles significantly decreased (indicated by arrows); the highlighted areas were analyzed and quantitated by area and pixel intensity. **A.** baseline wrinkles; **B.** relaxation and loss of wrinkles due to ISO treatment; **C.** baseline wrinkles upon thresholding; **D.** threshold of relaxation and wrinkle loss due to ISO treatment.

2.10 GPR30 protein expression in heart, aorta and VSMCs

Freshly isolated hearts and aortas from normotensive WKY and hypertensive SHR rats were washed with ice-cold PBS, transferred into lysis buffer, chopped into small pieces with dissecting scissors, homogenized using a disposable pestle system (Fisherbrand) and then centrifuged at 13000 rpm for 10 minutes, at 4˚C. The resultant supernatants were collected for western blotting. VSMCs were isolated from aorta of WKY rats as described in section 2.2. Cells (passages 0-2) were washed with PBS and lysed with ice-cold lysis buffer. Proteins were separated by SDS-PAGE, transferred to PVDF membrane and immunoblotted for GPR30 content as we recently described (Gros et al, 2011a). GPR30 antibody (1:500) was obtained from MBL International (Woburn, MA).

2.11 Assessment of vascular reactivity in aortic rings

To investigate the potential vasodilatory role of GPR30, I examined its vascular reactivity in aortic rings according to previously published methods (Gros et al, 1994, Liu et al, 2003), WKY rats were anesthetized, the chest wall was opened, and a portion of aorta was removed quickly to a Petri dish containing ice-cold Krebs physiological salt solution (KPSS) of the following composition (mM): NaCl 118.0, $NaHCO_3$, 25.0, d-glucose 11.1, KC1 4.72, $CaCl_2$ 2.56, NaH_2PO_4 1.13, and $MgCl_2$ 1.12. (-)Ascorbic acid (0.114 mM) and disodium EDTA (0.0297 mM) were added to stabilize test drugs.

Blood and connective tissue were removed carefully, and four ring segments (3

mm wide) from each aorta were cut and suspended in KPSS under optimal tension (0.75 g) in double-walled organ baths maintained at 37℃ and gassed with 5% CO_2 and 95% O_2 individually. The rings were equilibrated for 60 minutes, during which time they were maximally constricted with phenylephrine (PE, final bath concentration, 10 μM) twice, KPSS was changed three times after each dose and the tension was adjusted as necessary. In experiments with endothelium-denuded aortic rings, the endothelial layer was removed by gentle rubbing and flushing with distilled water. The absence of functional endothelium was inferred by the loss of methylcholine-mediated relaxation in PE-preconstricted rings. Tension generated was measured using isometric Force Displacement Transducers (Model FT03) and Compact Transducer Amplifiers (Model P11T, Grass Technologies, West Warwick, RI), which linked through a Data Acquisition System (DI-720 Series) to the computer. WinDaq Data Acquisition Software (DATAQ Instruments, Inc., Akron, OH) was used to record the waveform and analyze data.

Following the equilibration period, rings were submaximally constricted with PE (final bath concentration, 100 nM) and allowed to reach a plateau that remained stable for 15 minutes (baseline). After washout of PE, aortic rings were incubated in the presence or absence of 1 μM G15, and increasing concentrations of aldosterone (Aldo, 10 pM-100 nM) or G1 (1 nM-1 μM). G15 was added 15 minutes before the addition of PE. Aldo or G1 was added 2 minutes before PE. In each experiment, one or two rings were assessed after sequential cycles of PE-mediated constriction without other treatment and thus served as temporal controls. PE-mediated constriction was

quantified by determining the area under the curve (AUC) using a trapezoidal method of analysis (*Figure 2.3*). The effects of G15, Aldo or G1 on PE-mediated contraction responses were expressed as a percentage of baseline response normalized for temporal changes as assessed in the parallel control ring segments.

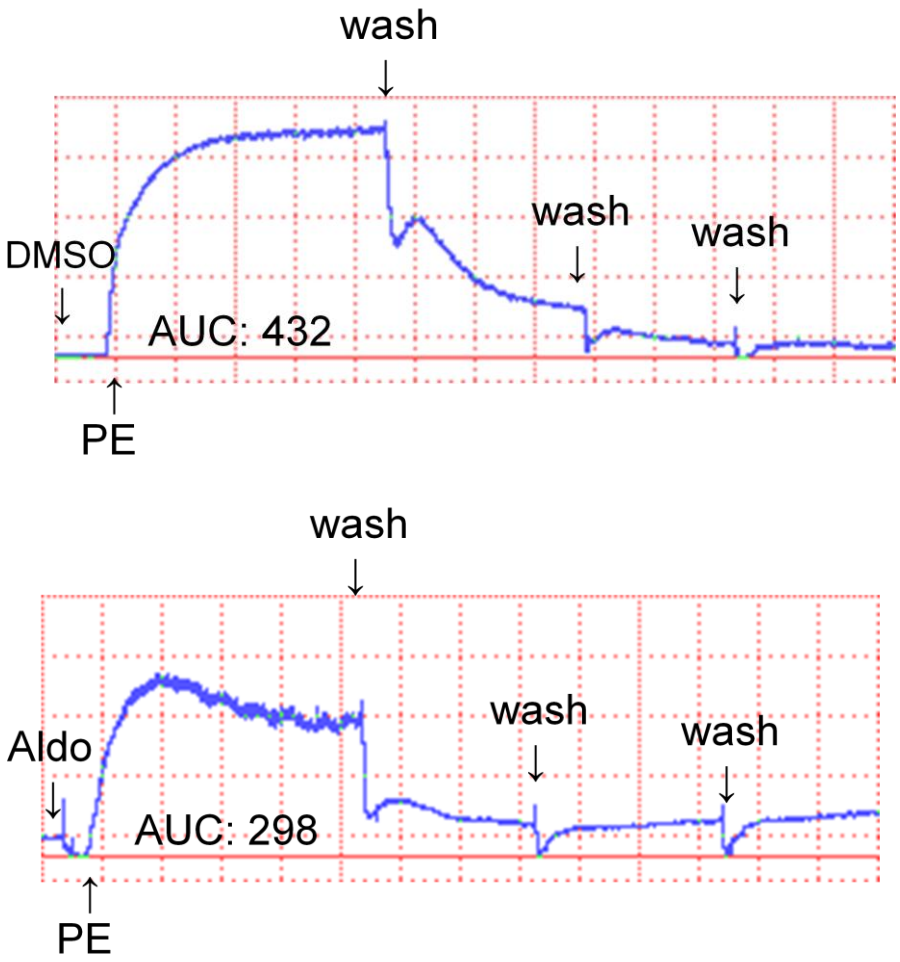

Figure 2.3. Representative tracings from control and aldosterone-treated aortic ring segments. Aldo attenuated PE-mediated constriction in endothelium-intact aortic ring segments. Constriction response with PE was quantified by determination of AUC using trapezoidal method of analysis.

2.12 Wound healing assay

To determine the effect of GPR30 on cell migration and proliferation, I performed a wound healing assay. The wound healing assay has been carried out for many years to estimate the migration and proliferation rates of different cell types and under differant culture conditions (Denker & Barber, 2002). ECs or VSMCs were infected with GFP, SiGFP, GPR30 or SiGPR30 adenovirus as described in section 2.5. Forty-eight hours post-infection, cells were nearly confluent (~90%) in 35 mm dishes. A sterile 10 μl pipet tip was used to scratch wounds through the cells perpendicular to a line drawn on the bottom of the dish (*Figure 2.4*). After changing the medium, cells were pretreated in the presence or absence of the GPR30 antagonist G15, and then treated with vehicle or GPR30 agonist aldosterone, estradiol or G1. Wound area was then inspected microscopically and pictures were taken every 8 or 24 hours. The open gaps (wounds) were calculated as percentage of basal area using image analysis software (Northern Eclipse 6.0; Empix Imaging, Toronto, ON).

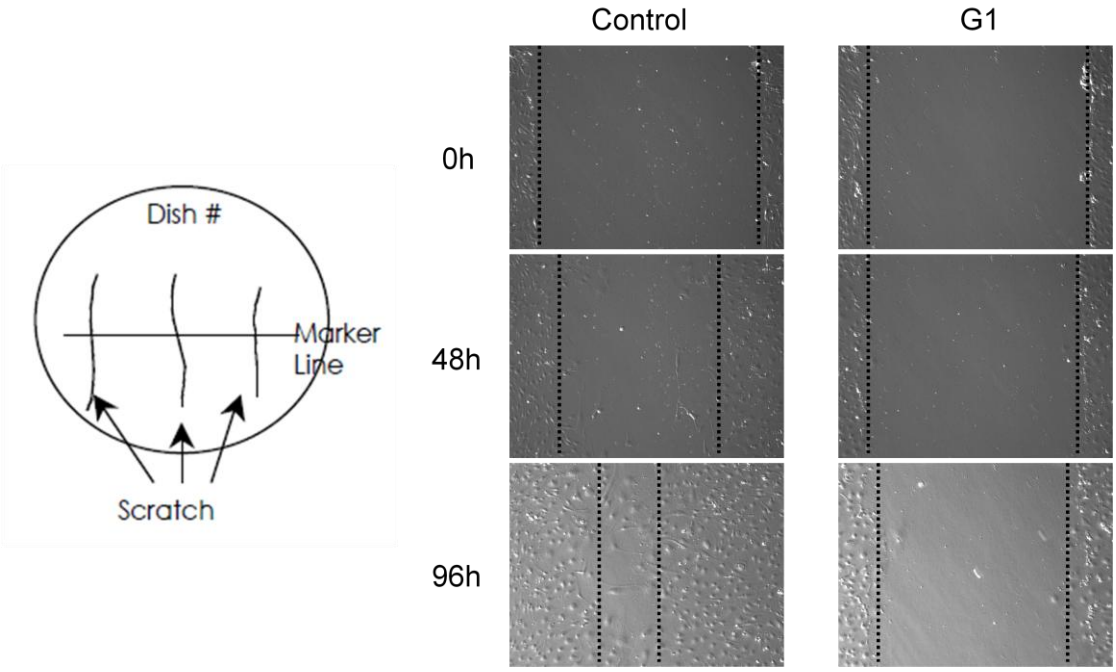

Figure 2.4. Wound healing assay was used to estimate the migration and proliferation rates of cultured cells. Aortic ECs or SMCs from WKY rats were nearly confluent (~90%) in 35 mm dishes. A sterile 10 µl pipet tip was used to scratch wounds through the cells perpendicular to a line drawn on the bottom of the dish. Cells were infected with adenovirus or treated with different drugs (e.g. G1). Wound area was then inspected microscopically and pictures were taken at the indicated time points. The open gaps (wounds) were calculated as percentage of basal area using image analysis software (Northern Eclipse 6.0; Empix Imaging, Toronto, ON).

2.13 Immunofluorescence assay

To investigate whether GPR30 can co-localize with GRK2, I performed double immunofluorescence experiment. VSMCs isolated from WKY and SHR rats were cultured in 60 mm dishes. Some dishes were infected with FLAG-tagged GPR30 adenovirus for 4 hours. Twenty-four hours post-infection, cells were seeded onto 35 mm glass bottom microwell dishes at 30-50% confluency. After incubation in the presence or absence of 10 pM Aldo or 1 μM G1 for 15 minutes, cells were fixed using 2% paraformaldehyde for 15 minutes, permeabilized in 100% methanol for 10 minutes, and subsequently blocked with 3% BSA for 1 hour to reduce nonspecific staining. Cells were then incubated with mouse-anti-FLAG (1:500, Sigma-Aldrich, Oakville, ON) and rabbit-anti-GRK2 antibodies (1:500, Santa Cruz Biotechnology, Santa Cruz, CA) at 4℃ overnight. Following washes with PBS, cells were probed with AlexaFluor 488 goat-anti rabbit and AlexaFluor 594 goat-anti mouse secondary antibodies (1:1000, Invitrogen) for 1 hour at room temperature in the dark. DAPI was used to detect nuclei. As a negative control, the primary antibody was omitted from the staining procedure. Finally, cells were visualized through a Zeiss LSM 410 confocal microscope equipped with a Krypton / Argon laser using a 63× oil immersion lens.

2.14 Co-immunoprecipitation of GPR30 and GRK2

To determine whether GPR30 interacts with GRK2, I performed co-IP experiment. VSMCs isolated from WKY rats were infected with adenovirus encoding GFP or FLAG-tagged GPR30. The media was changed once after 24 hours. Forty-eight hours post-infection, cells were treated in the presence or absence of 10 pM Aldo or 1 μM

G1 for 15 minutes and then harvested. After washing with PBS and spinning, the cell pellet was resuspended in lysis buffer. The samples were centrifuged at 13000 rpm for 10 minutes and the supernatants were transferred to new microcentrifuge tubes. 500 µl of supernatant was incubated with 50 µl of a 50% slurry of lgG agarose beads (equilibrated in lysis) for 1 hour and subjected to centrifugation and then transferred into fresh tubes. Pre-cleared cell lysates (500 µl) were incubated with 10 µg anti-FLAG antibody and 50 µl of lgG agarose beads overnight at 4°C, with gentle rotation. Cell lysates were then subjected to centrifugation and agarose beads were washed by resuspension and centrifugation three times in lysis buffer. Proteins were released from the beads by heating at 99 ℃ for 5 minutes, subsequent to the addition of loading buffer. Proteins were separated by SDS-PAGE, transferred to PVDF membrane and immunoblotted for GRK2 content. As a negative control, each lysate was incubated with purified agarose beads as appropriate to determine non-specific binding. To verify protein expression, 5% of each cell lysate taken prior to the pull-down experiment was assessed by immunoblotting in parallel with immunoprecipitated samples.

2.15 Statistical analyses

For multiple group comparisons, an initial analysis by ANOVA was followed by Dunnett's multiple comparison test. The significance of difference for between unpaired groups was determined by Student's t test for unpaired data. A value for $P < 0.05$ on a two-sided test was taken as the minimum level of significance (Prism 4.0; GraphPad Software Inc., San Diego, CA).

2.16 References

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CHAPTER 3

RESULTS

3 RESULTS

3.1 GRK2 protein expression in cultured VSMCs

To determine GRK2 protein expression levels in cultured VSMCs from WKY and SHR rats, I performed immunoblotting. GRK2 protein was detected at approximately 80 kilodaltons (*Figure 3.1A*). The expression of GRK2 protein in VSMCs from SHRs was significantly elevated by 50±7% compared to normotensive WKY rats ($p<0.05$, *Figure 3.1B*). This result is consistent with our previous data in human hypertension (Gros et al, 1997, Gros et al, 1999) as well as animal models (Gros et al, 2000), which suggests an association between increased GRK2 expression and hypertension, confirming that the phenotype persisted in cultured VSMCs.

3.2 Alteration of GRK2 protein expression by adenoviral infection

To alter GRK2 protein expression levels, I utilized adenoviral constructs to infect VSMCs. To determine the infection efficiency of our adenoviral constructs, WKY and SHR VSMCs were infected with increasing concentrations of GRK2 or SiGRK2 adenovirus. GFP and SiGFP adenovirus constructs were used as controls. GRK2 expression was enhanced by increasing concentrations of GRK2 virus in both WKY and SHR VSMCs (*Figure 3.2*), whereas increasing concentrations of SiGRK2 virus led to attenuation of GRK2 expression (*Figure 3.3*). Western blots were used to determine the virus concentration needed to increase or decrease GRK2 expression levels by approximately 50%.

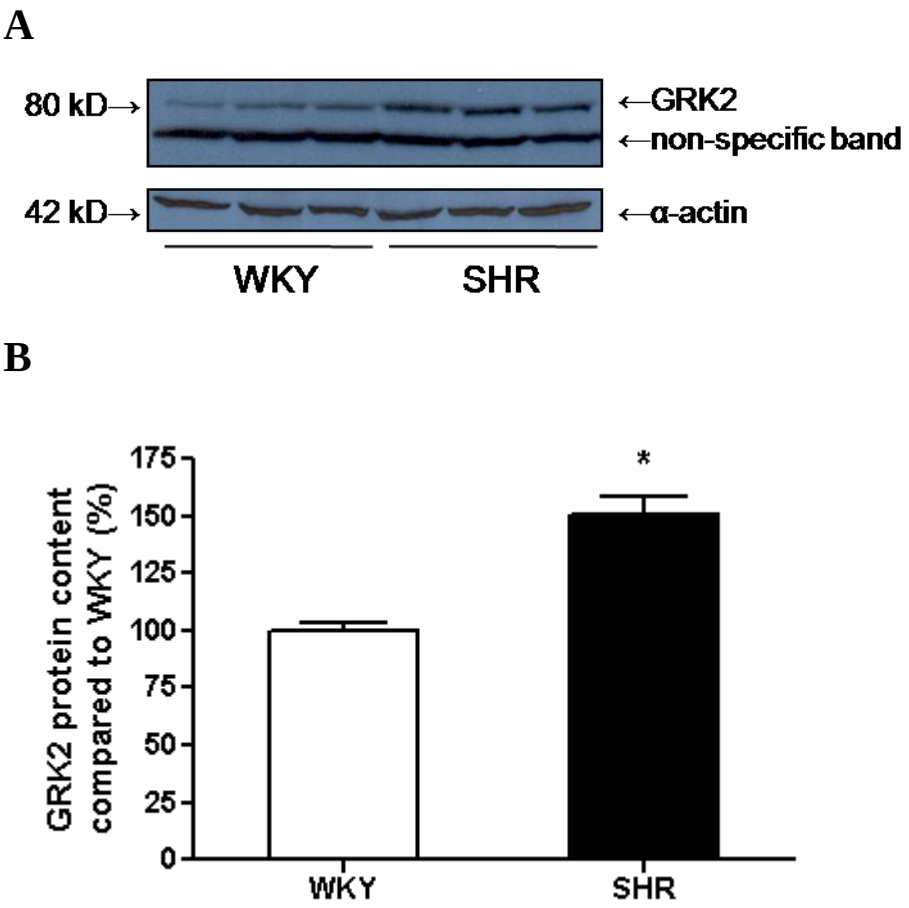
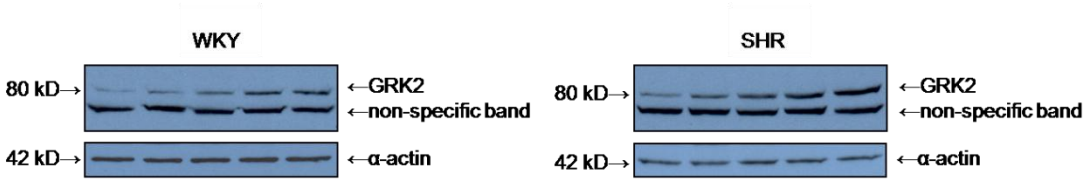


Figure 3.1. GRK2 protein expression in cultured VSMCs from WKY and SHR rats. Cells were washed with PBS and lysed with ice-cold lysis buffer. 50 μg of protein were loaded on a 10% SDS-PAGE. After transfer, the PVDF membrane was in turn incubated with a 1:1000 GRK2 antibody and a 1:5000 anti-rabbit secondary antibody. GRK2 protein bands were then detected by ECL assay. **A.** GRK2 protein was detected at approximately 80 kilodaltons. **B.** Densitometric analysis of SHR GRK2 content was expressed as a percentage of GRK2 in WKY rats. *P<0.05 vs. WKY. Data represent means ± SEM from 3 independent experiments performed under identical conditions.

A



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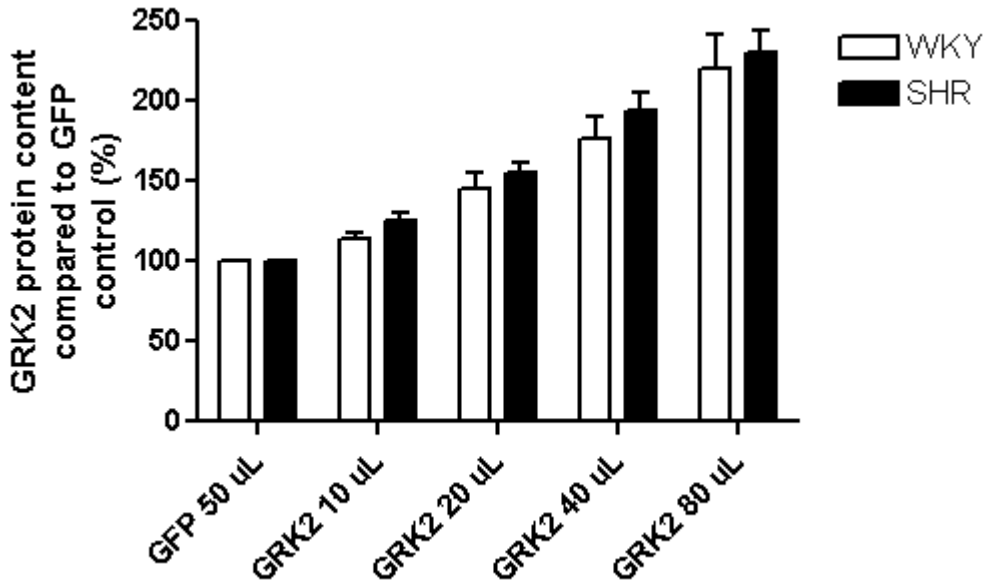
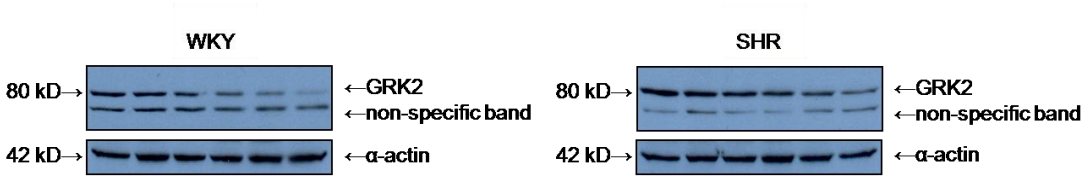


Figure 3.2. GRK2 protein expression in WKY and SHR VSMCs infected by increasing concentrations of GRK2 adenovirus. Cells were infected by increasing concentrations of GRK2 adenovirus, GFP virus was used as control. Medium was changed once after 24 hours. VSMCs were lysed 48 hours post-infection and used for Western blotting. **A.** GRK2 protein was detected at approximately 80 kilodaltons. **B.** Densitometric analysis of GRK2 content was expressed as a percentage of GFP control, respectively. Data represent means ± SEM from 4 independent experiments performed under identical conditions.

A



B

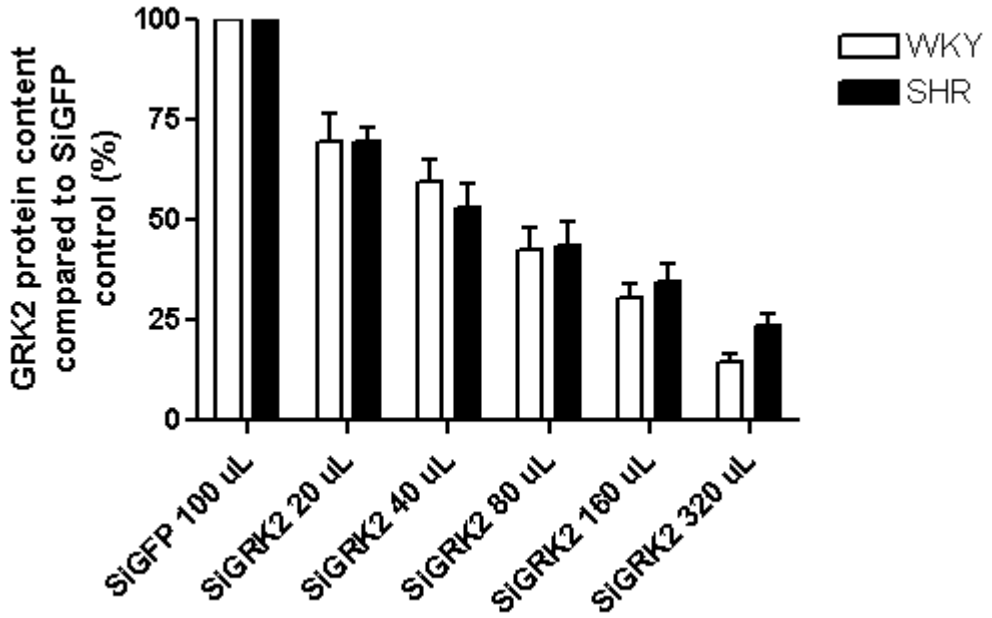


Figure 3.3. GRK2 protein expression in WKY and SHR VSMCs infected by increasing concentrations of SiGRK2 adenovirus. Cells were infected by increasing concentrations of SiGRK2 adenovirus, SiGFP virus was used as control. Medium was changed once after 24 hours. VSMCs were lysed 48 hours post-infection and used for Western blotting. **A.** GRK2 protein was detected at approximately 80 kilodaltons. **B.** Densitometric analysis of GRK2 content was expressed as a percentage of GFP control, respectively. Data represent means ± SEM from 4 independent experiments performed under identical conditions.

3.3 The effects of altering GRK2 protein expression levels on G_q-mediated signaling in VSMCs from WKY and SHR rats

To examine the effects of altering GRK2 protein expression levels on G_q-mediated ERK activation, I examined the short-term (5 minutes) effects of angiotensin II and vasopressin. The effects of these hormones on ERK activation were compared with that of platelet-derived growth factor (PDGF, hormone acting through a tyrosine kinase receptor pathway, as opposed to a GPCR pathway). Both WKY and SHR VSMCs were infected with GRK2 or SiGRK2 adenovirus to achieve a 50% change in GRK2 protein expression level. Western blot results showed that all treatments significantly increased phospho-ERK1/2. Treatment with angiotensin II (*Figure 3.4*) and vasopressin (*Figure 3.5*) both led to significant enhancements in phospho-ERK levels in SHR cells compared to WKY cells. In addition, GRK2 over-expression greatly increased ERK activation in both cells. On the other hand, knocking down GRK2 resulted in a reduction of ERK phosphorylation. However, PDGF-induced ERK activation was not altered by changing GRK2 content (*Figure 3.6*). Taken together, these results suggested that altering GRK2 expression led to a corresponding change in certain vasoconstrictor mediated G_q signaling.

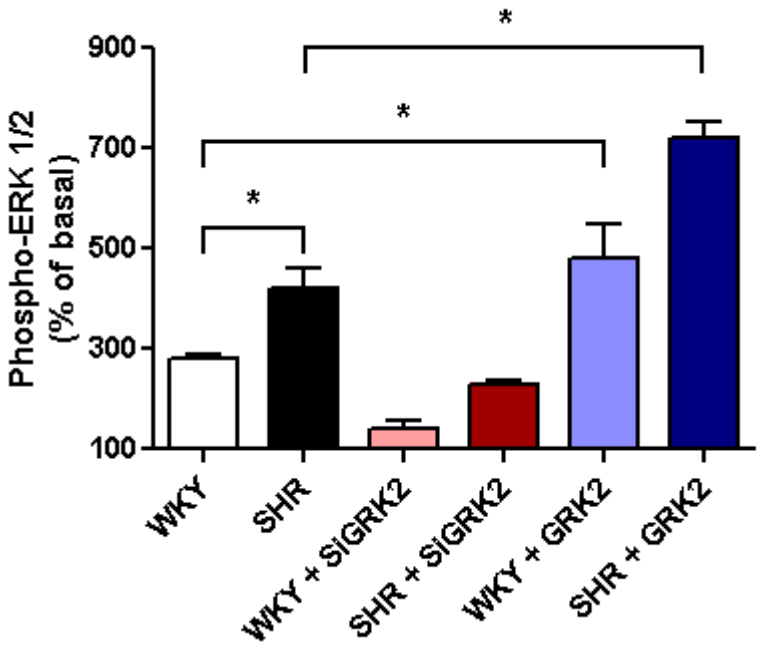


Figure 3.4. Short-term effect of angiotensin II on mitogen-activated protein kinase activity as assessed by phospho-ERK. VSMCs were infected with GRK2 or SiGRK2 adenovirus. After serum starvation for 24 hours, cells were incubated in the presence or absence of 1 μM angiotensin II for 5 minutes and then lysed with ice-cold lysis buffer. Densitometric analysis of phospho-ERK1/2 content was normalized to their own basal (untreated) condition. Total-ERK1/2 was used as loading control. *P<0.05. Data represent the means ± S.E.M. from 3 to 6 independent experiments performed under identical conditions.

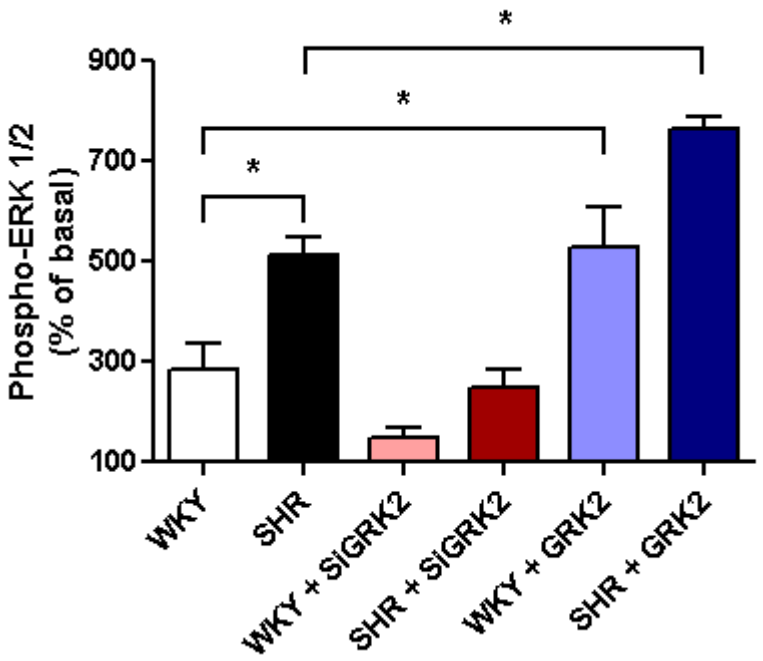


Figure 3.5. Short-term effect of vasopressin on mitogen-activated protein kinase activity as assessed by phospho-ERK. VSMCs were infected with GRK2 or SiGRK2 adenovirus. After serum starvation for 24 hours, cells were incubated in the presence or absence of 1 μM vasopressin for 5 minutes and then lysed with ice-cold lysis buffer. Densitometric analysis of phospho-ERK1/2 content was normalized to their own basal (untreated) condition. Total-ERK1/2 was used as loading control. **P*<0.05. Data represent the means ± S.E.M. from 3 to 6 independent experiments performed under identical conditions.

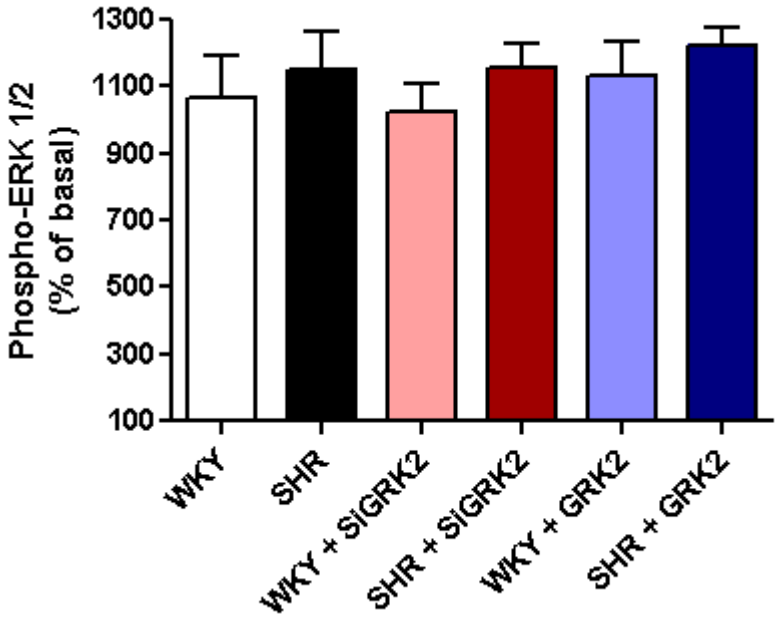


Figure 3.6. Short-term effect of PDGF on mitogen-activated protein kinase activity as assessed by phospho-ERK. VSMCs were infected with GRK2 or SiGRK2 adenovirus. After serum starvation for 24 hours, cells were incubated in the presence or absence of 20 ng/ml PDGF for 5 minutes and then lysed with ice-cold lysis buffer. Densitometric analysis of phospho-ERK1/2 content was normalized to their own basal (untreated) condition. Total-ERK1/2 was used as loading control. Data represent the means ± S.E.M. from 3 to 6 independent experiments performed under identical conditions.

3.4 The effects of altering GRK2 protein expression levels on G_s-mediated signaling in VSMCs from WKY and SHR rats

To assess the effects of over-expression or knocking down of GRK2 on G_s mediated signaling, I performed the arborization assay. In VSMCs, isoproterenol (ISO) mediates a progressive arborization process, characterized by a generalized retraction and an increase in central cellular volume. This effect can be mimicked by forskolin (FSK), which increases intracellular cAMP by a more direct effect on adenylyl cyclase activation. We evaluated arborization responses as a read out of β- adrenergic dependant cAMP production. Analogous to the impaired vasodilator effects of ISO reported previously in intact vessels from hypertensive animals and humans (Feldman & Gros, 1998), the ISO-mediated arborization response was significantly attenuated in VSMCs from SHRs compared with WKY rats ($p<0.05$, *Figure 3.7A*). In contrast, FSK-stimulated arborization was not significantly different between WKY and SHR (*Figure 3.7B*).

To further examine the effects of altering GRK2 expression levels on ISO-mediated arborization, VSMCs were infected with GFP, SiGFP, GRK2 or SiGRK2 adenovirus. Over-expression of GRK2 in WKY VSMCs impaired ISO-mediated arborization by 47% compared with GFP control ($p<0.05$, *Figure 3.8A*), whereas knocking down GRK2 expression in SHR VSMCs resulted in an improved ISO-mediated arborization (by 56%, $p<0.05$, *Figure 3.8B*). These results demonstrated that increased GRK2 expression attenuated certain vasodilator mediated G_s signaling.

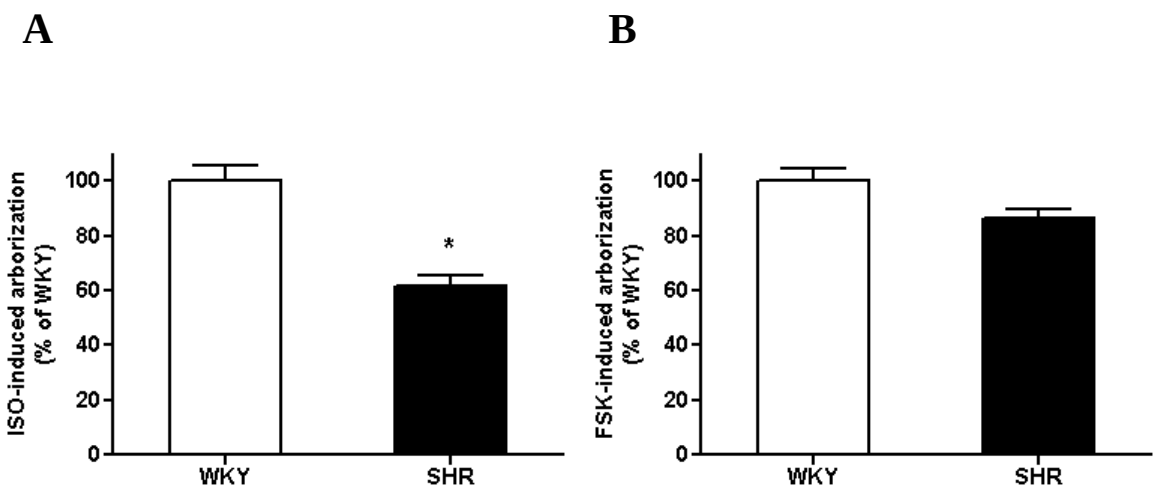


Figure 3.7. Isoproterenol- and forskolin-induced arborization in VSMCs from WKY rats and SHRs. VSMCs were cultured on 35-mm dishes. After serum starvation for 24 hours, cells were incubated for 15 minutes in a temperature controlled chamber maintained at 37℃ on an inverted microscope, then treated with either **A.** 10 μM ISO or **B.** 10 μM FSK. The progression of the arborization response was measured in the dark using time-lapse video microscopy. The extent of arborization was assessed by determining the change in image intensity using the threshold setting within the image analysis software. The change in image intensity at 40 minute time point was expressed as a percentage of basal intensity (before the addition of drug). **P*<0.05 vs. WKY. Data represent the means ± S.E.M. from 4 independent experiments performed under identical conditions.

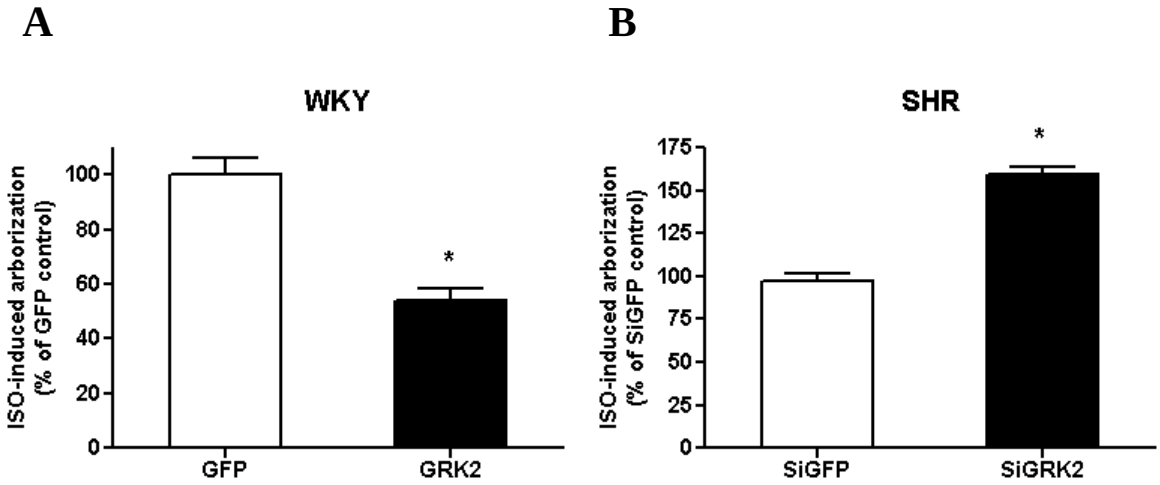


Figure 3.8. The effects of altering GRK2 expression levels on isoproterenol-mediated arborization. **A.** WKY VSMCs were cultured onto 35-mm dishes, infected with GFP or GRK2 adenovirus for 24 hours before being serum-starved for another 24 hours. Cells were incubated for 15 minutes in a temperature controlled chamber maintained at 37℃ on an inverted microscope, then treated with 10 μM ISO. The extent of arborization was assessed by determining the change in image intensity using the threshold setting within the image analysis software. The change in image intensity at the 40 minute time point was expressed as a percentage of basal intensity (before the addition of drug). **P*<0.05 vs. GFP control. **B.** SHR VSMCs were cultured onto 35-mm dishes, infected with SiGFP or SiGRK2 adenovirus for 24 hours before being serum-starved for another 24 hours. The same protocol was followed as described above. **P*<0.05 vs. SiGFP control. Data represent the means ± S.E.M. from 4 independent experiments performed under identical conditions.

3.5 The effects of altering GRK2 protein expression levels on WKY VSMC contractility

To determine the contractile reactivity of cultured VSMCs, I utilized a silicone wrinkle assay as previously described (Boswell et al, 1992, Gros et al, 2011a, Harris et al, 1980). To assess GPCR-mediated vasodilation, via activation of G_s, the dose response profile of the β-adrenergic receptor agonist, ISO (0.1 nM-10 μM) was assessed. This vasodilator response was compared to FSK-mediated vasodilation (0.1 nM-10 μM), which reflected a direct activation of adenylyl cyclase. VSMCs isolated from WKY rats were infected with GFP, GRK2 or SiGRK2 adenovirus. ISO-mediated relaxation was impaired following GRK2 over-expression as compared to GFP-infected cells, whereas SiGRK2 gene-transfer did not further enhance ISO-mediated vasodilation (*Figure 3.9*). In addition, the extent of FSK-stimulated VSMC relaxation was not significantly changed by GRK2 over-expression as compared to GFP- or SiGRK2- infected VSMCs (*Figure 3.10*). These results suggested that increased GRK2 expression attenuated G_s-mediated vasodilation in cultured VSMCs.

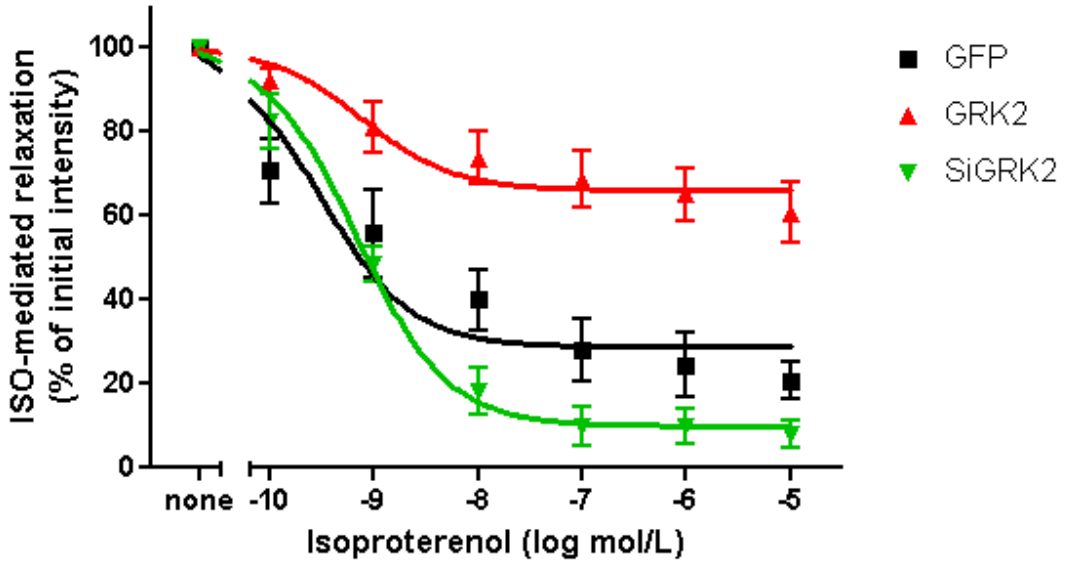


Figure 3.9. Isoproterenol mediated dose-dependent relaxation in VSMCs isolated from WKY rats. WKY VSMCs were infected with GFP, SiGRK2 or GRK2 adenovirus. 24 hours after infection, they were subcultured onto 35 mm dishes containing siliconized cover slips. Dishes were mounted in a temperature- controlled chamber at 37 ℃ on an inverted microscope. After 10 minutes of baseline recording, increasing doses of ISO were given to VSMCs at 5 minutes intervals. Contractile responses were assessed using time-lapse video microscopy. Wrinkle area was quantified using bright image thresholding, where the relaxation was reflected as decreased intensity. Data are representative of means ± S.E.M. from 4-9 independent experiments performed under identical conditions. Over-expression of GRK2 impairs ISO-mediated relaxation.

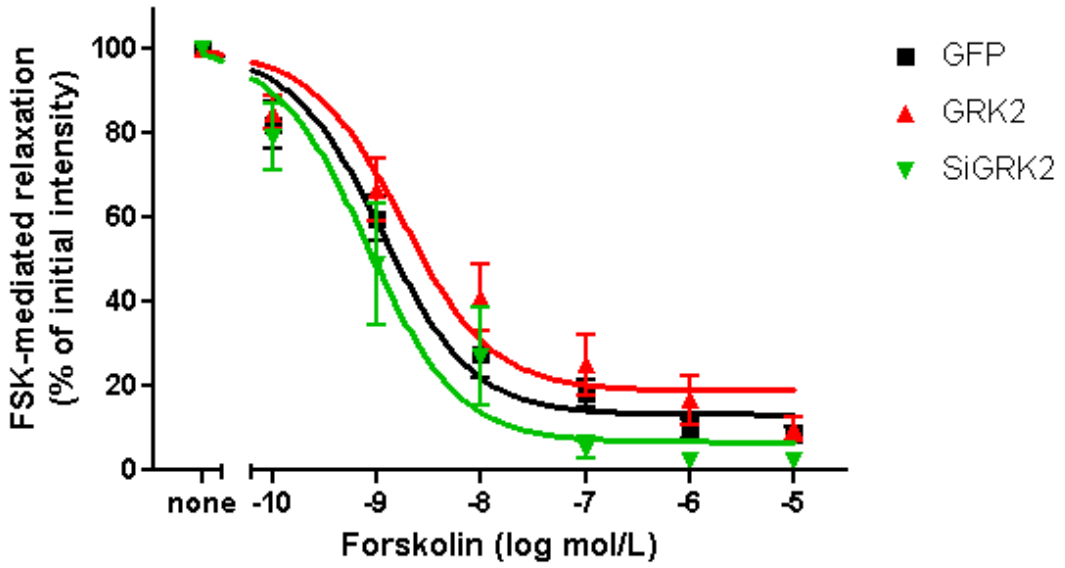


Figure 3.10. Forskolin mediated dose-dependent relaxation in VSMCs isolated from WKY rats. WKY VSMCs were infected with GFP, SiGRK2 or GRK2 adenovirus. 24 hours after infection, they were subcultured onto 35 mm dishes containing siliconized cover slips. Dishes were mounted in a temperature- controlled chamber at 37 ℃ on an inverted microscope. After 10 min of baseline recording, increasing doses of FSK were given to VSMCs at 5 min intervals. Contractile responses were assessed using time-lapse video microscopy. Wrinkle area was quantified using bright image thresholding, where the relaxation was reflected as decreased intensity. Data are representative of means ± S.E.M. from 4-11 independent experiments performed under identical conditions.

Taken together, the above data suggested that GPCR-mediated G_q and G_s signaling can be regulated by altering GRK2 expression. This may contribute to the regulatory role of GRK2 in hypertension. The primary function of GRK2 is phosphorylating activated receptors and in turn leading to receptor desensitization and internalization. It has been suggested that estradiol and aldosterone have rapid vascular effects during hypertension, which are in part mediated by a recently identified GPCR, named GPR30. Here I investigate the effect of GPR30 in aortic function upon different agonist treatments, and potential regulation of GPR30 by GRK2.

3.6 GPR30 protein expression in heart and aorta tissues as well as cultured VSMCs

To determine the expression level of GPR30 in hearts and aortas from WKY and SHR rats, isolated tissues were homogenized in lysis buffer and the resultant supernatants were collected for immunoblotting. GPR30 protein content in hearts (*Figure 3.11 A, B*) and aortas (*Figure 3.11 C, D*) obtained from SHRs was significantly reduced as compared to WKY rats. Interestingly, this was opposite to the expression pattern of GRK2 in these animals. Thus we hypothesize that GPR30 mediated cell signaling may be impaired in SHRs and this may related to the up-regulation of GRK2.

In regard to cultured cells, our previous studies have showed that persistent

expression of GPR30 was detectable by RT-PCR in cultured vascular ECs (Gros et al, 2011b), but declined in cultured VSMCs (Ding et al, 2009, Gros et al, 2011b). I also examined the GPR30 protein expression in cultured VSMCs. As demonstrated in *Figure 3.12*, GPR30 was expressed and detected in freshly isolated VSMCs (passage 0, day 0). However, it was lost in culture (P0, day 4 and 6, as well as P1 and P2).

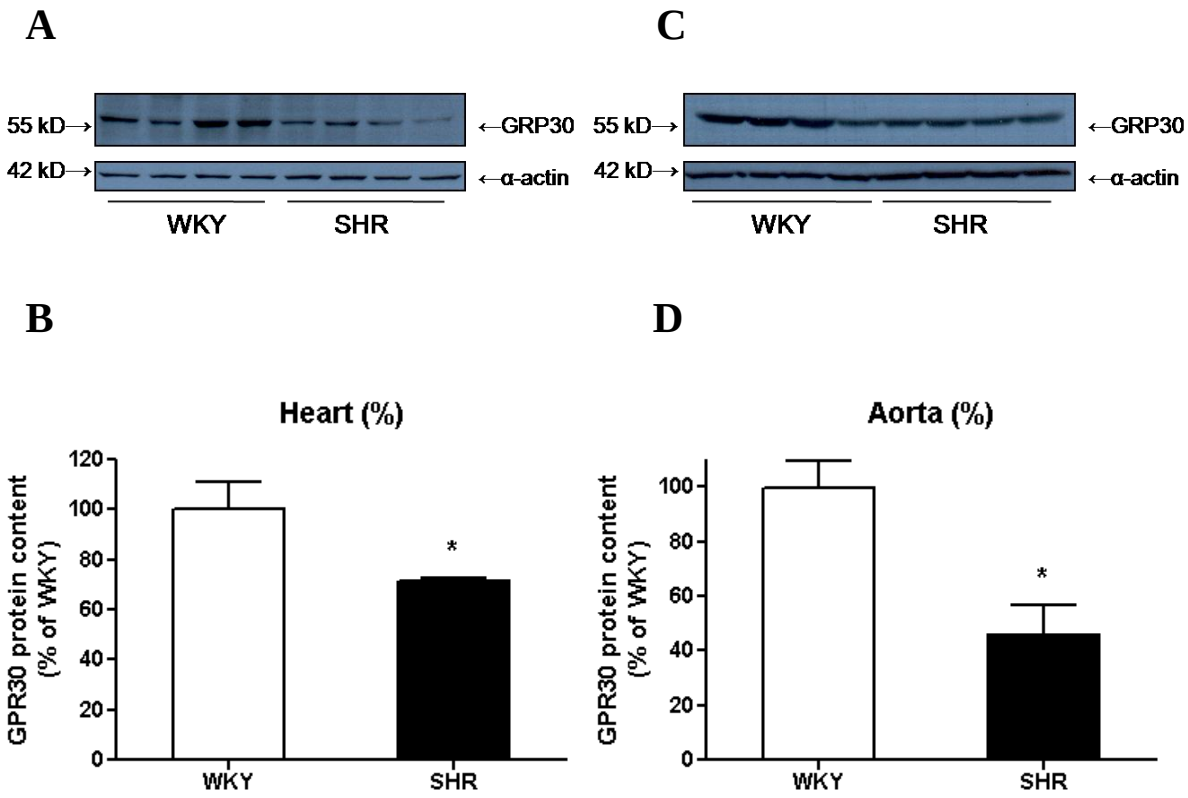


Figure 3.11. Assessment of GPR30 protein expression in isolated hearts and aortas from WKY and SHR rats. Freshly isolated hearts and aortas were washed with ice-cold PBS, transferred into lysis buffer, chopped into small pieces with dissecting scissors, homogenized using a disposable pestle system and then centrifuged at 16000xg for 10 minutes, at 4˚C. The resultant supernatants were collected for western blotting. Proteins were separated by SDS-PAGE, transferred to PVDF membrane and immunoblotted for GPR30 content. **A.** Representative immunoblot of GPR30 expression in hearts. **B.** Densitometric analysis of GPR30 content expressed in hearts as a percentage of WKY. **C.** Representative immunoblot of GPR30 expression in aortas. **D.** Densitometric analysis of GPR30 content expressed in aortas as a percentage of WKY. **P*<0.05 vs. WKY. Data represent the mean ± S.E.M. from 4 independent experiments performed under identical conditions.

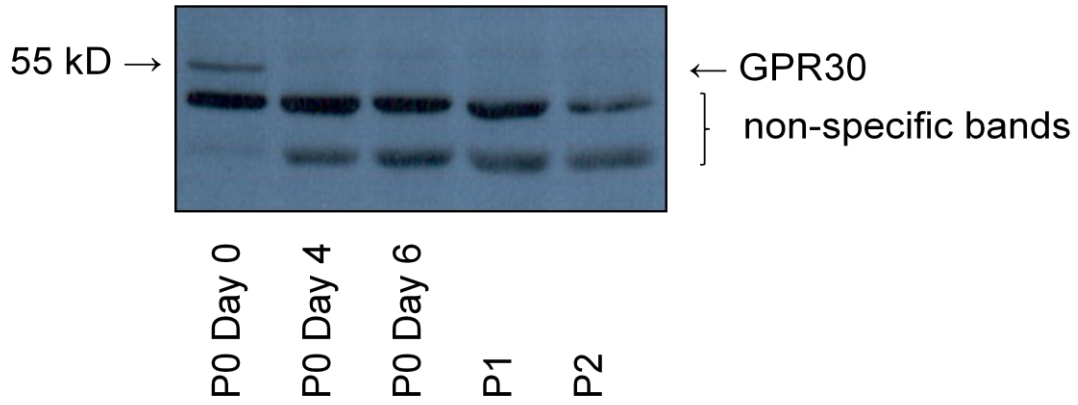


Figure 3.12. GPR30 protein expression in cultured VSMCs from WKY rats. VSMCs were isolated from aorta of WKY rats. Indicated passages of cells were washed with PBS and lysed with ice-cold lysis buffer. Proteins were separated by SDS-PAGE, transferred to PVDF membrane and immunoblotted for GPR30 content. GPR30 protein was detected at approximately 55 kilodaltons. GPR30 was expressed in freshly isolated VSMCs (passage 0, day 0). However, it was lost in culture (P0, day 4 and 6, as well as P1 and P2).

3.7 Assessment of GPR30 mediated vascular reactivity in aortic rings

Furthermore, I examined the potential effect of GPR30 activation on vascular reactivity. Aortic rings from WKY rats were cut and suspended in KPSS under optimal tension (0.75g) in individually double-walled organ baths maintained at 37℃ and gassed with 5% CO_2 and 95% O_2. The constriction response to phenylephrine (PE) was quantified by determination of the area under the curve as described in methods. Aldosterone (Aldo) and G1 both appeared to act as agonists of GPR30. As shown in *Figure 3.13 and 3.15*, Aldo and G1 both caused attenuation of PE-mediated vasoconstriction in aortic ring segments. G15 has been suggested to be a GPR30 antagonist. 1 μM G15 alone had no effect on this PE-mediated constriction, but it inhibited the vasodilatory effect of 10 pM Aldo (*Figure 3.14*). Interestingly, in contrast, G15 did not block the effect of G1 (*Figure 3.16*). I further examined the effect of G15 and G1 in endothelium-denuded aortic rings. G15 alone had a very weak vasoconstrictor effect, and the vasodilatory effect of G1 was attenuated compared with endothelium-intact rings. Moreover, this G1 effect did not seem to be affected by G15. (*Figure 3.17*).

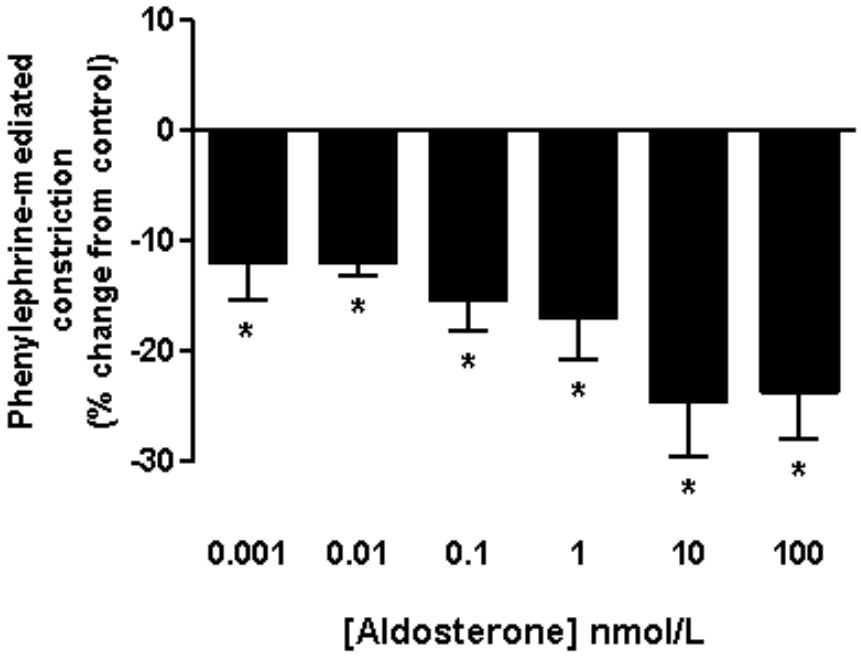


Figure 3.13. Aldosterone attenuates phenylephrine-mediated constriction in endothelium-intact aortic ring segments. Aortic rings were submaximally constricted with 100 nM PE and allowed to reach a plateau that remained stable for 15 minutes (baseline). After washout of PE, aortic rings were incubated in the indicated concentrations of Aldo. Aldo was added 2 minutes before PE. Two of four rings were assessed after sequential cycles of PE-mediated constriction without other treatment and thus served as temporal controls. PE-mediated constriction was quantified by determining the area under the curve generated by the data acquisition software. The effects of Aldo on PE-mediated contraction responses were expressed as a percentage of the baseline response normalized for temporal changes as assessed in the parallel control ring segments. Data represent means ± SEM for 5 independent experiments. **P*<0.05 vs. control, ANOVA; Dunnett's multiple comparison test.

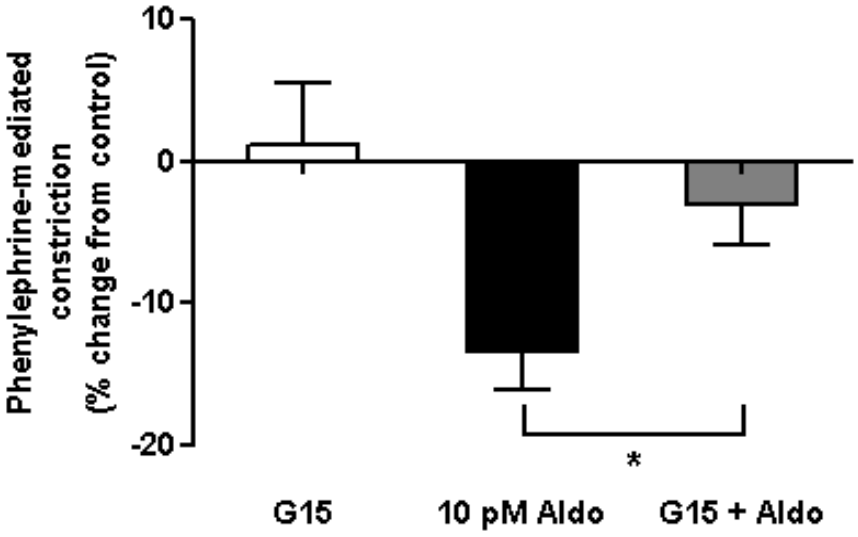


Figure 3.14. G15 blocked aldosterone-induced attenuation of phenylephrine-mediated constriction in endothelium-intact aortic ring segments. After baseline recording, aortic rings were incubated in the presence or absence of 1 μM G15, and 10 pM of Aldo. G15 was added 15 minutes before the addition of PE. Aldo was added 2 minutes before PE. One of four rings was assessed after sequential cycles of PE-mediated constriction without other treatment and thus served as temporal control. PE-mediated constriction was quantified by determining the area under the curve. The effects of G15 and Aldo on PE-mediated contraction responses were expressed as a percentage of the baseline response normalized for temporal changes as assessed in the parallel control ring segment. *P<0.05, Data represent means ± SEM for 6 independent experiments.

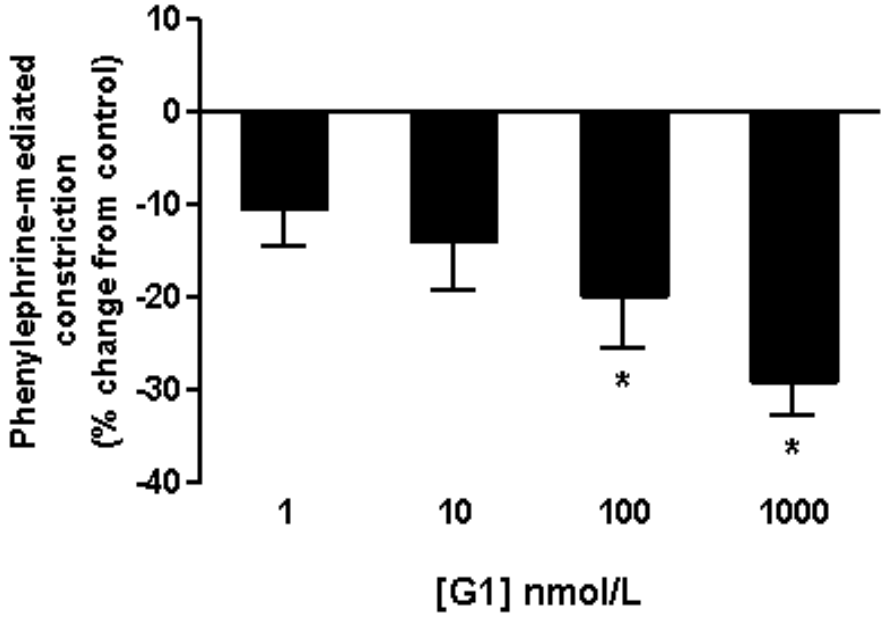


Figure 3.15. G1 attenuates phenylephrine-mediated constriction in endothelium-intact aortic ring segments. After baseline recording, aortic rings were incubated in the indicated concentrations of G1. G1 was added 2 minutes before PE. Two of four rings were assessed after sequential cycles of PE-mediated constriction without other treatment and thus served as temporal controls. PE-mediated constriction was quantified by determining the area under the curve generated by the data acquisition software. The effects of G1 on PE-mediated contraction responses were expressed as a percentage of baseline response normalized for temporal changes as assessed in the parallel control ring segments. Data represent means ± SEM for 5 independent experiments. *P<0.05 vs. control, ANOVA; Dunnett's multiple comparison test.

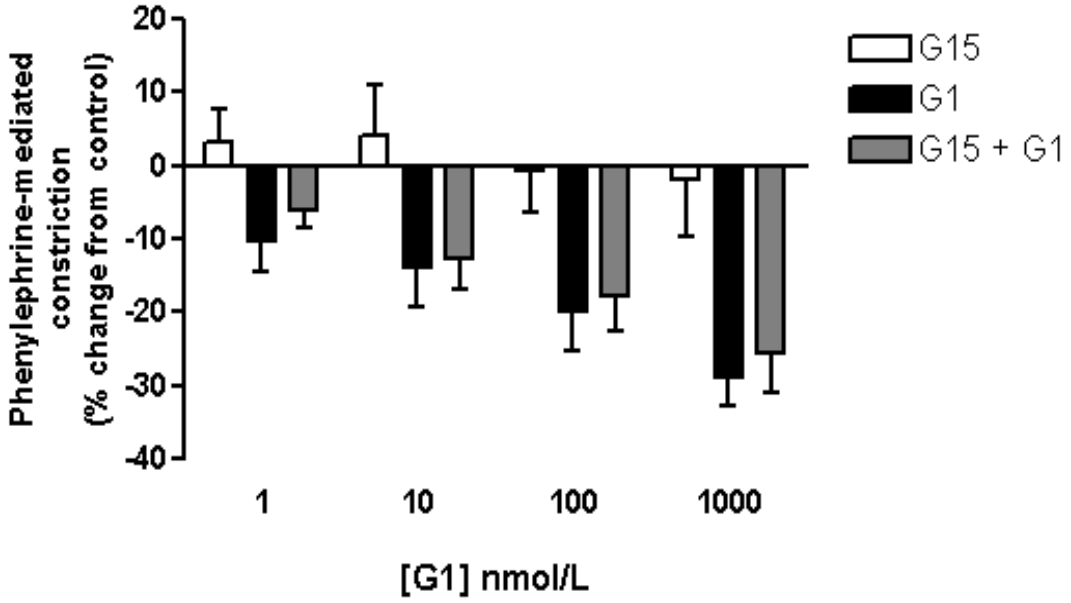


Figure 3.16. G15 did not block G1-induced attenuation of phenylephrine-mediated constriction in endothelium-intact aortic ring segments. After baseline recording, aortic rings were incubated in the presence or absence of 1 μM G15, and the indicated concentrations of G1. G15 was added 15 minutes before the addition of PE. G1 was added 2 minutes before PE. One of four rings was assessed after sequential cycles of PE-mediated constriction without other treatment and thus served as temporal control. PE-mediated constriction was quantified by determining the area under the curve generated by the data acquisition software. The effects of G15 and G1 on PE-mediated contraction responses were expressed as a percentage of baseline response normalized for temporal changes as assessed in the parallel control ring segment. Data represent means ± SEM for 5 independent experiments.

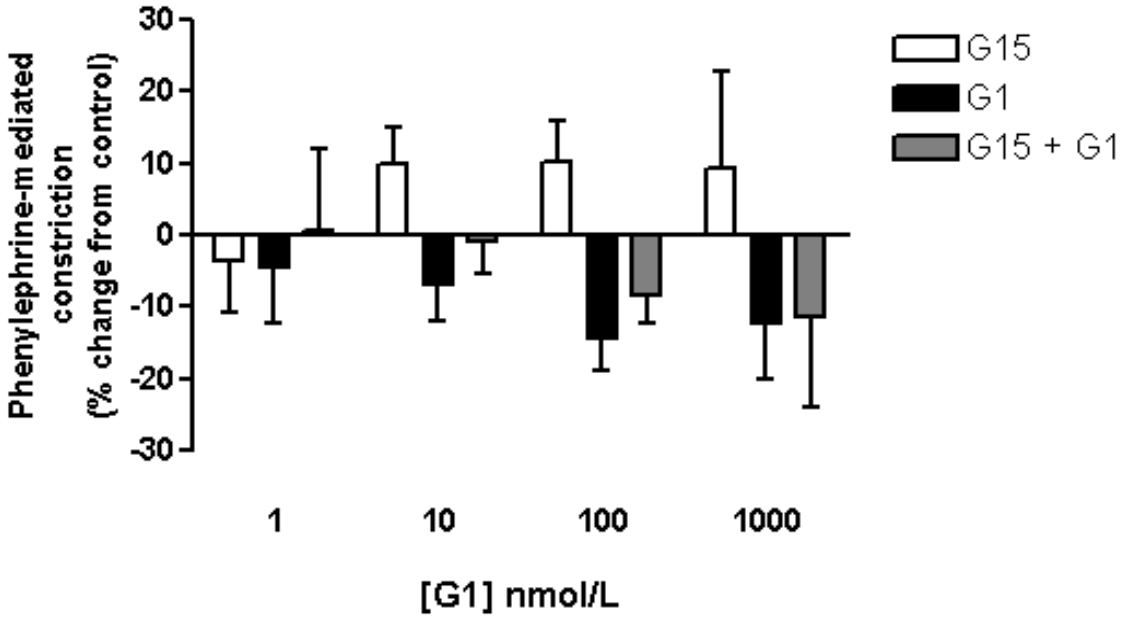


Figure 3.17. The effect of G15 and G1 in endothelium-denuded aortic rings. The endothelial layer was removed by gentle rubbing and flushing with distilled water. The absence of functional endothelium was inferred by the loss of methylcholine-mediated relaxation in PE-preconstricted rings. After baseline recording, aortic rings were incubated in the presence or absence of 1 μM G15, and the indicated concentrations of G1. G15 was added 15 minutes before the addition of PE. G1 was added 2 minutes before PE. One of four rings was assessed after sequential cycles of PE-mediated constriction without other treatment and thus served as temporal control. PE-mediated constriction was quantified by determining the area under the curve generated by the data acquisition software. The effects of G15 and G1 on PE-mediated contraction responses were expressed as a percentage of baseline response normalized for temporal changes as assessed in the parallel control ring segment. Data represent means ± SEM for 5 independent experiments.

3.8 The effects of GPR30 agonist in cultured EC

Our previous study (Liu et al, 2003) and current data suggested that Aldo and G1 caused different responses in endothelium denuded rings compared with endothelium intact rings. I further examined the effect of different GPR30 agonists in cultured ECs. It has been suggested that GPR30 is related to EC growth and apoptosis (Gros et al, 2011b). Thus, I utilized a wound healing assay to estimate the effect of GPR30 activation on EC migration and proliferation. As shown in *Figure 3.18*, neither 10 nM estrodial nor 10 pM aldosterone produced any appreciable effect in this assay. However, wound healing of ECs was much slower when treated with 1 μM G1, and surprisingly this effect was not blocked by 1 μM G15. To investigate if this G1 effect was dose-dependent, I examined the effect of increasing doses of G1 (0.03 μM-3 μM) on wound healing in the presence or absence of 1 μM G15 pretreatment (*Figure 3.19 A, B*). Wound healing of ECs is relatively sensitive to G1, within the 0.1-1 μM range, and G15 did not block the effects of G1 (*Figure 3.19 C*). These data indicated that the effect of G1 on ECs proliferation might be GPR30 independent. I also knocked down GPR30 protein expression in ECs using SiGPR30 adenovirus. As demonstrated in *Figure 3.20*, knocking down GPR30 expression in ECs did not attenuate the G1 effect. To further examine the specificity of this G1 effect in different cells and confirm the independency of this effect from GPR30, I performed parallel experiments using VSMCs. 1 μM G1 did not alter the wound healing, and GPR30 over-expression did not enhance the effect of G1 (*Figure 3.21*). All these results together reinforced the idea that this G1 effect on wound healing was GPR30 independent and EC specific.

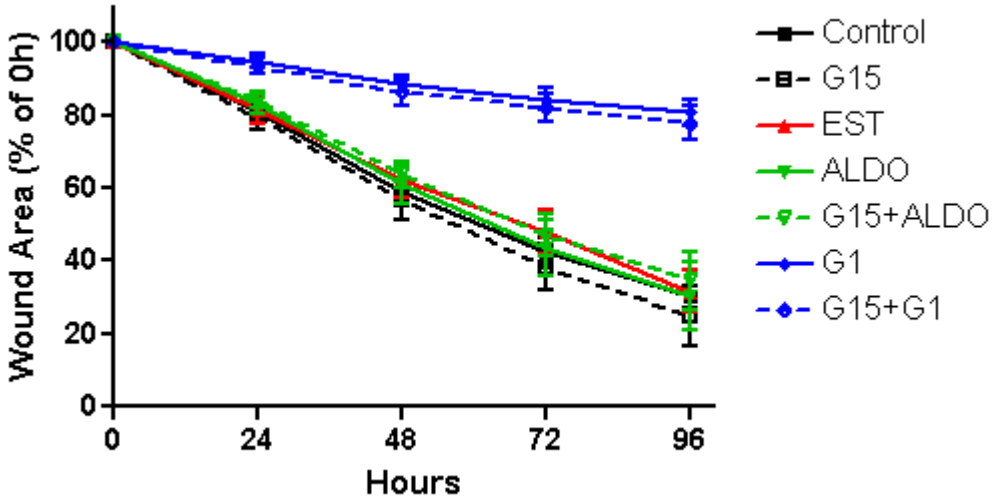


Figure 3.18. Wound healing of ECs in response to estradiol, aldosterone or G1 (with or without G15 pretreatment). Cells were nearly confluent (~90%) in 35 mm dishes. A sterile 10 µl pipet tip was used to scratch a wound through the cells perpendicular to a line drawn on the bottom of the dish. After changing the medium, cells were pretreated in the presence or absence of the GPR30 antagonist G15 (1 µM), and then treated with vehicle or GPR30 agonist estradiol (10 nM), aldosterone (10 pM) or G1 (1 µM). Wound area was then inspected microscopically and pictures were taken every 24 hours. The open gaps were calculated as percentage of basal area using image analysis software. Data represent means ± SEM for 5 independent experiments.

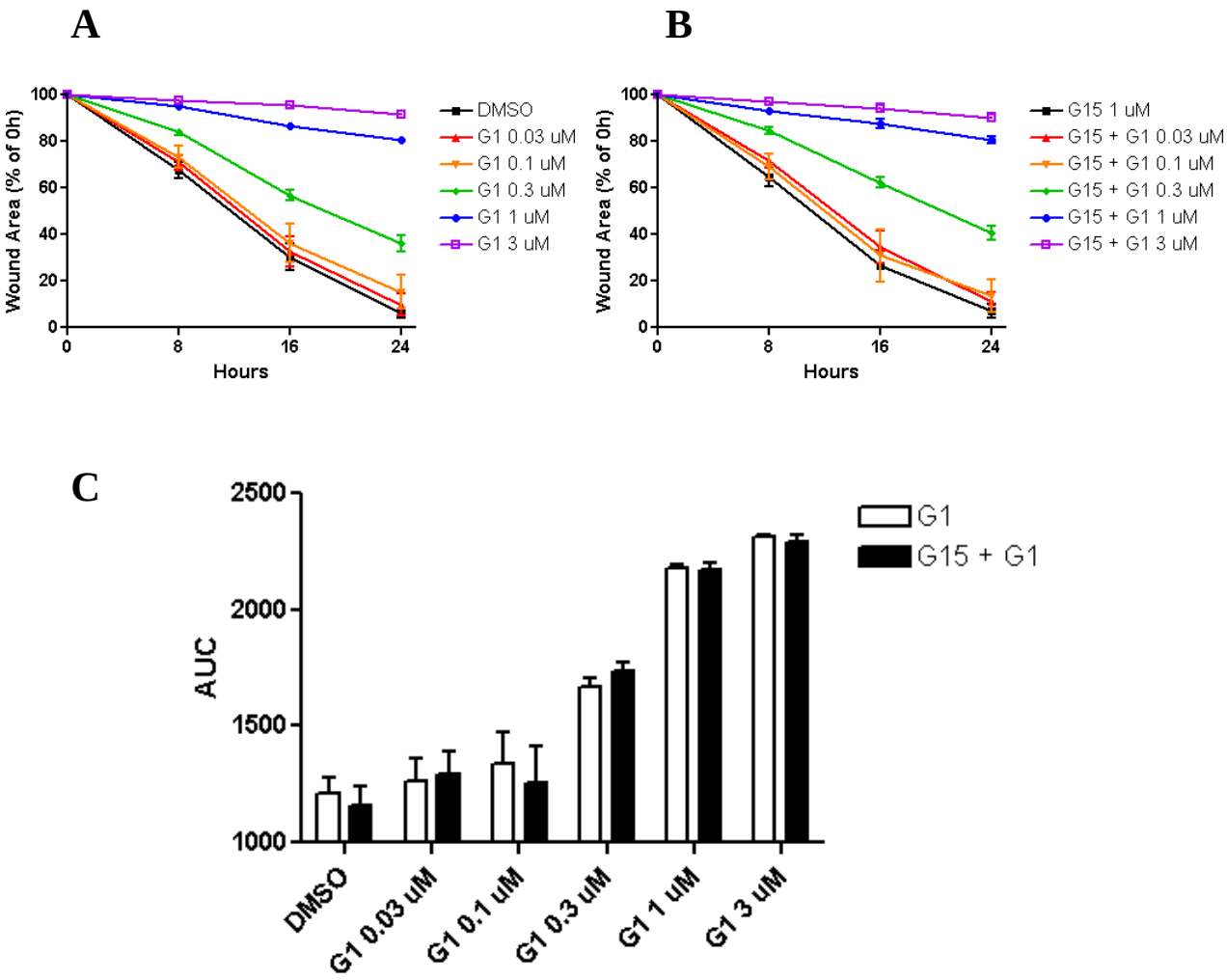


Figure 3.19. Wound healing of ECs in response to increasing doses of G1 (with or without G15 pretreatment). Cells were nearly confluent (~90%) in 35 mm dishes. A sterile 10 µl pipet tip was used to scratch a wound through the cells perpendicular to a line drawn on the bottom of the dish. **A**. After changing the medium, cells were treated with vehicle or the indicated doses of G1 and wounds were assessed as described in methods. **B**. Cells were pretreated with 1 µM G15, and then treated with vehicle or the indicated doses of G1 and wounds were assessed as described in methods. **C**. Data obtained in these experiments were calculated as area under the curve. Pretreatment of G15 did not block the effect of G1. Data represent means ± SEM for 4 independent experiments.

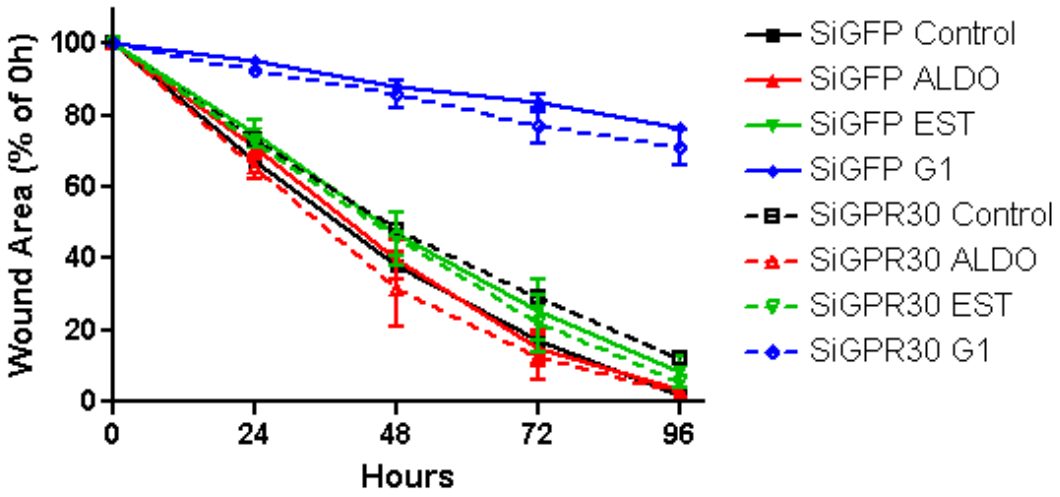


Figure 3.20. Wound healing of ECs in response to aldosterone, estradiol or G1 (with or without SiGPR30 virus infection). ECs were infected with SiGFP or SiGPR30 adenovirus. Forty-eight hours post-infection, cells were nearly confluent (~90%) in 35 mm dishes. A sterile 10 µl pipet tip was used to scratch a wound through the cells perpendicular to a line drawn on the bottom of the dish. After changing the medium, cells were treated with vehicle or GPR30 agonist aldosterone (10 pM), estradiol (10 nM) or G1 (1 µM). Wound area was then inspected microscopically and pictures were taken every 24 hours. The open gaps were calculated as percentage of basal area using image analysis software. Data represent means ± SEM for 3 independent experiments.

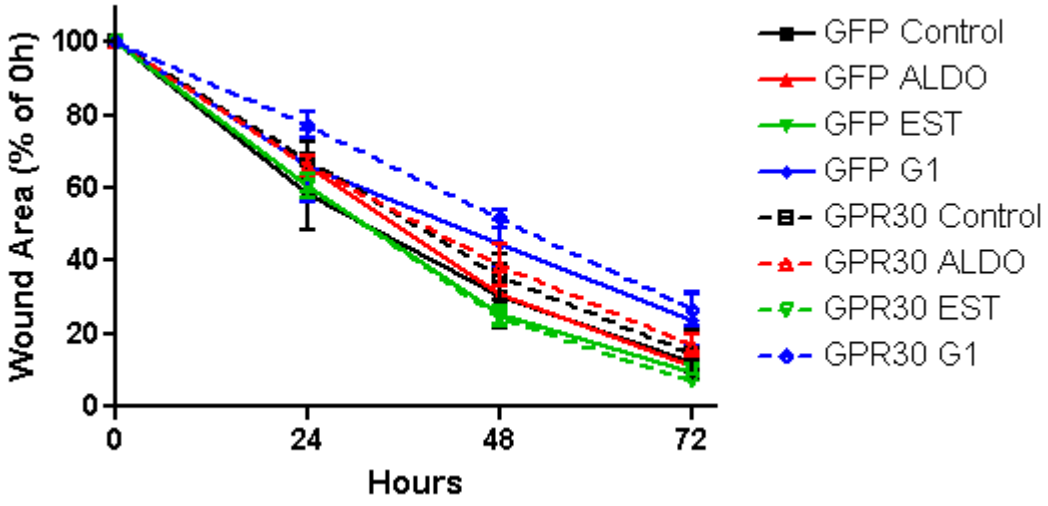


Figure 3.21. Wound healing of VSMCs in response to aldosterone, estradiol or G1 (with or without GPR30 virus infection). VSMCs were infected with GFP or GPR30 adenovirus. Forty-eight hours post-infection, cells were nearly confluent (~90%) in 35 mm dishes. A sterile 10 µl pipet tip was used to scratch a wound through the cells perpendicular to a line drawn on the bottom of the dish. After changing the medium, cells were treated with vehicle or GPR30 agonist aldosterone (10 pM), estradiol (10 nM) or G1 (1 µM). Wound area was then inspected microscopically and pictures were taken every 24 hours. The open gaps were calculated as percentage of basal area using image analysis software. Data represent means ± SEM for 3 independent experiments.

In total, we have studied the effect of GPR30 in both aortic rings and cultured vascular cells. Earlier data have shown that the expression of GPR30 is reduced in tissues from SHRs. One possible interpretation of the down-regulation of GPR30 in SHRs is elevated receptor internalization and degradation. Here, we examine the possibility that GRK2 is able to phosphorylate GPR30 and regulate its desensitization, internalization and degradation.

3.9 Localization of GRK2 and GPR30 in VSMCs

To investigate whether GPR30 can co-localize with GRK2, I performed double immunofluorescence assay. Since GPR30 expression is greatly diminished in cultured VSMCs, I reintroduced GPR30 by infecting VSMCs with FLAG-tagged GPR30 adenovirus. Cells were visualized through a Zeiss LSM 410 confocal microscope equipped with a Krypton / Argon laser using a 63× oil immersion lens. GRK2 was located uniformly in the cytosol, while GPR30 was more localized to the nucleus. After being treated with 1 pM aldosterone or 1 μM G1, more GPR30 transferred to the cell membrane. However, little or no co-localization between GPR30 and GRK2 was observed under basal or stimulated condition (*Figure 3.22*).

3.10 Potential interaction between GRK2 and GPR30 in VSMCs

To further explore the possibility of interaction between GRK2 and GPR30, a co-IP experiment was performed. VSMCs isolated from WKY rats were infected with adenovirus encoding GFP or FLAG-tagged GPR30. Following IP with anti-FLAG, no GRK2 protein was detected, which suggested that there was no interaction between GRK2 and GPR30 in cultured VSMCs (*Figure 3.23*).

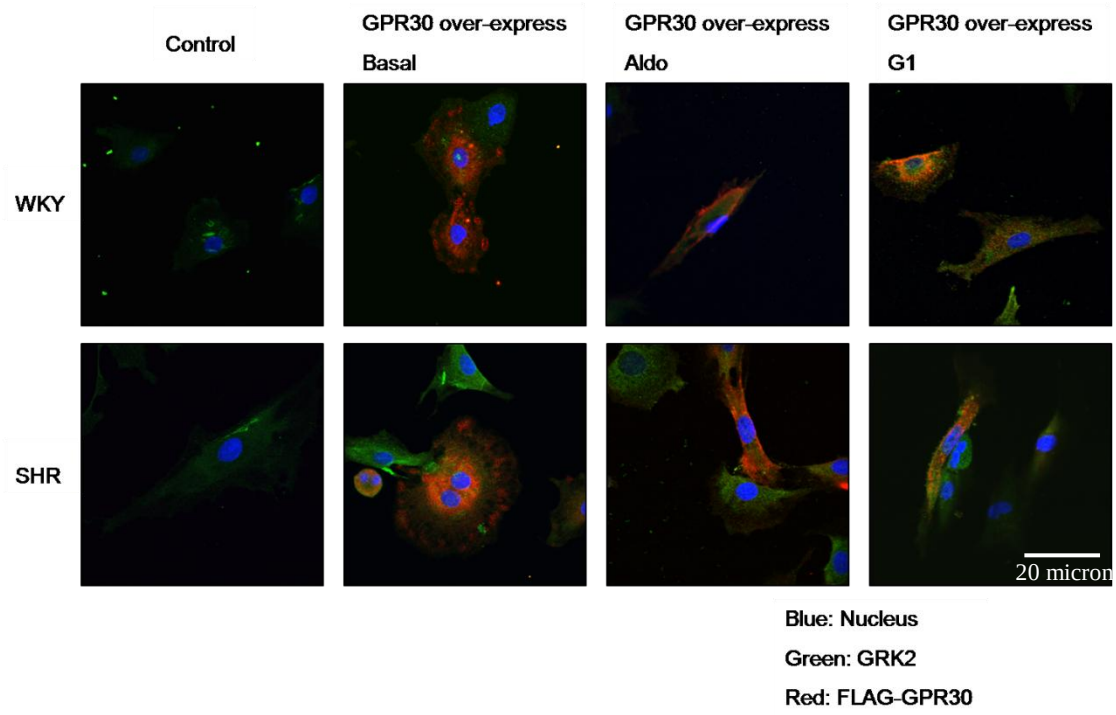


Figure 3.22. Localization of GRK2 and GPR30 in VSMCs from WKY and SHR rats. VSMCs isolated from WKY and SHR rats were cultured in 60 mm dishes. Some dishes were infected with FLAG-tagged GPR30 adenovirus for 4 hours. Twenty-four hours post-infection, cells were seeded onto 35 mm glass bottom microwell dishes at 30-50% confluency. After incubation in the presence or absence of 10 pM Aldo or 1 μM G1 for 15 minutes, cells were fixed, permeabilized and then labeled with mouse-anti-FLAG and rabbit-anti-GRK2 antibodies. Following washes with PBS, cells were probed with AlexaFluor 488 goat-anti-rabbit and AlexaFluor 594 goat-anti-mouse secondary antibodies. DAPI was used to detect nuclei. Cells were visualized through a Zeiss LSM 410 confocal microscope equipped with a Krypton / Argon laser using a 63× oil immersion lens.

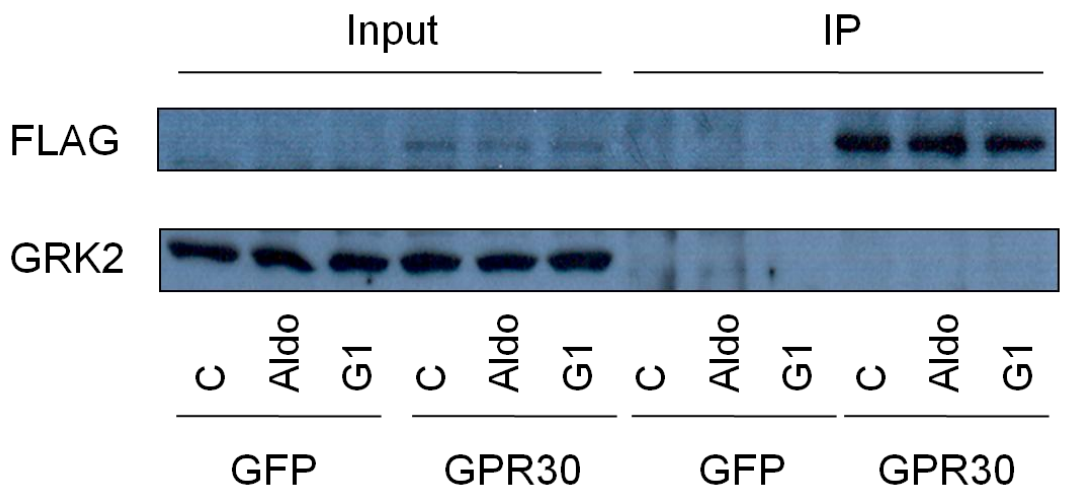


Figure 3.23. Protein co-immunoprecipitation of VSMC lysates from WKY rats. VSMCs isolated form WKY rats were infected with adenovirus encoding GFP or FLAG-tagged GPR30. Forty-eight hours post-infection, cells were treated in the presence or absence of 1 pM Aldo or 1 μM G1 for 15 minutes and then lysed with ice-cold lysis buffer. Pre-cleared cell lysates (500 μl) were incubated with 10 μg anti-FLAG antibody and 50 μl lgG agarose beads overnight at 4°C, with gentle rotation. Cell lysates were then subjected to centrifugation and agarose beads were washed by resuspension and centrifugation three times in lysis buffer. Proteins were released from the beads by heating at 99 ℃ for 5 minutes, subsequent to the addition of loading buffer. Proteins were separated by SDS-PAGE, transferred to PVDF membrane and immunoblotted for GRK2 content. To verify protein expression, 5% of each cell lysate taken prior to the pull-down experiment was assessed by immunoblotting in parallel with immunoprecipitated samples.

3.11 References

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CHAPTER 4

DISCUSSION

4 DISCUSSION

4.1 Hypertension and GRK2

Previous studies in both human and animal models have confirmed that with regard to short-term effects on vascular reactivity, the hypertensive state is characterized by impairment in vasodilator mechanisms (acting through GPCRs linked to G_s/adenylyl cyclase activation) (Feldman & Gros, 1998). We have also demonstrated that the growth regulatory effects of β-adrenoceptor activation (the prototype GPCR linked to G_s/adenylyl cyclase) are comparably impaired with regard to β-adrenergic-mediated effects on ERK activation (Gros et al, 2006).

β-adrenergic-mediated vasodilation and β-adrenergic-stimulated adenylyl cyclase activation are impaired in hypertension. These effects have been demonstrated in both human and experimental models of hypertension and both in vivo and in vitro (Feldman & Gros, 1998). My studies have confirmed that this functional impairment in β-adrenergic-mediated responses is still apparent in cultured single cell vascular systems in spontaneously hypertensive rats, a commonly used genetic model of hypertension. It is noteworthy that analogous "durability" of altered regulation of adenylyl cyclase in hypertension has been reported in "immortalized" lymphoblasts from hypertensive patients (Siffert et al, 1995). The persistence of this "phenotype" of impaired β-adrenergic responses in cultured cell systems would suggest that these defects are not due to short-term in vivo regulatory effects in hypertension, which

would be expected to be diminished and ultimately be undetectable when assessed in vitro in cultured cells with ongoing passaging.

My current studies also demonstrate that the expression of GRK2 protein in VSMCs from SHRs was significantly elevated compared to normotensive WKY rats (Figure 3.1). This result is consistent with our previous data in human hypertension (Gros et al, 1997, Gros et al, 1999) as well as animal models (Gros et al, 2000), which suggested an association between increased GRK2 expression and hypertension, confirming that this phenotype remained in these cultured VSMCs.

Having confirmed that the increased GRK2 expression levels are maintained in cultured VSMCs from SHRs, I examined if these GRK levels could be manipulated in cultured VSMCs using over-expression and knockdown (SiRNA) techniques in conjunction with adenoviral constructs. Using both WKY and SHR VSMCs I was able to precisely increase and decrease the GRK2 protein content on demand (Figures 3.2 and 3.3). Using this adenoviral approach I was able to either increase or decrease the GRK2 expression levels (by approximately 50%) similarly to what we had observed in comparing the WKY versus SHR VSMCs. Previous studies have demonstrated impaired ISO-mediated ERK activation in VSMCs from SHRs as compared to WKY (Gros et al, 2006). Therefore, I next examined the effects of increasing or decreasing GRK2 expression levels on ERK activation by vasopressin, angiotensin II (acting via G_q-activation) as well as PDGF. It was clear that under endogenous GRK2 expression levels that both vasopressin- and angiotensin II-mediated ERK activation responses were enhanced in VSMCs obtained from SHRs

(Figures 3.4 and 3.5). This effect seems to be GPCR-selective, since PDGF-mediated responses (not acting via GPCRs) were not altered when comparing WKY versus SHR VSMCs. Similarly GRK2 over-expression greatly increased ERK activation in both type of cells (Figures 3.4 and 3.5). On the other hand, knocking down GRK2 resulted in a reduction of ERK phosphorylation (Figures 3.4 and 3.5). Taken together, these results suggested that altering GRK2 expression led to a corresponding change in certain vasoconstrictor mediated G_q signaling. In addition, manipulation of GRK2 expression levels can clearly alter the phenotype of the VSMCs. That is, increasing GRK2 expression levels in WKY cells makes them appear similar in vasopressin and angiotensin II responses as unaltered SHR VSMCs and similarly knocking down GRK2 in SHR VSMCs (back to levels similar to WKY) makes them have responses that are like unaltered WKY VSMCs (Figures 3.4 and 3.5). All these manipulations appear to be GPCR-specific since PDGF-mediated ERK activations were not significantly altered by increasing or decreasing GRK2 expression levels in either cell type (Figure 3.6).

To further examine the potential of increasing or decreasing GRK2 expression levels in isolated cultured VSMCs, I utilized the arborization assay and silicone wrinkle assay and the responses of vasodilator mediated G_s signaling pathways. I observed that isoproterenol-mediated arborization was significantly attenuated in VSMCs from SHRs as compared to WKY VSMCs (Figure 3.7a). In contrast, forskolin-stimulated arborization was not significantly different between WKY and SHR (Figure 3.7b). Altering the GRK2 expression levels, could either impair

ISO-mediated arborization in GRK2 over-expressing WKY VSMCs, whereas knocking down GRK2 expression using SiRNA in SHR VSMCs resulted in an improved ISO-mediated arborization (Figure 3.8). Similarly, in the wrinkle assay, ISO-mediated relaxation was impaired following GRK2 over-expression (Figure 3.9) while FSK-stimulated VSMC relaxation (Figure 3.10) was not altered by the over-expression of GRK2.

Taken together, GPCR-mediated G_q and G_s signaling can be regulated by acutely altering GRK2 expression. This may contribute to the regulatory role of GRK2 in hypertension which is a developmental disease (i.e. with developmental aging). Nevertheless, the ultimate goal of this series of studies was to utilize adenoviral constructs to manipulate the GRK2 expression in isolated aortic ring segments obtained from WKY and SHR rats using an organ culture approach. Unfortunately in many of our preliminary experiments we were unable to get either sufficient protein expression and/or tissue viability following the adenoviral infections and therefore abandoned this line of research. Although, manipulation of GRK2 expression in an acute setting may someday become viable for the vasculature in the setting of hypertension, GRK2 will probably remain a potential future target with novel therapeutics (small molecules, stem cells, etc). Indeed in the setting of heart failure great progress has been made with the use of altering GRK2 expression and disease outcome using small molecule approaches.

4.2 GPR30, hypertension and GRK2

I also examined the potential role of a newly discovered GPCR, named GPR30, in vascular reactivity, wound healing and the possible interaction with GRK2. The interest in GPR30 has grown substantially in recent years. This was in part due to its initial discovery as a G protein-coupled receptor for steroid hormones especially estrogen (hence the suggested name change to GPER1, G protein estrogen receptor-1), but also more recently for aldosterone. In addition, we were interested in studying this receptor due to the link between aldosterone and estrogen with cardiovascular disease.

Our previous studies demonstrated that aldosterone and estrogen both stimulate vascular smooth muscle cell contraction via PI3 kinase-mediated mechanisms and that aldosterone can mediate vasodilation via an endothelial-dependent pathway (Liu et al, 2003). We have also delineated that these effects can be mediated by their classical receptors, MR and ER, and importantly, that the effects of both estradiol and aldosterone are enhanced by the expression of GPR30, a dynamically regulated receptor (Gros et al, 2011a). My initial studies focused on the expression levels of GPR30 in tissues obtained from 10 week old normotensive WKY and hypertensive SHRs. I demonstrated that GPR30 protein content in hearts and aortas obtained from SHRs was significantly reduced as compared to WKY rats (Figure 3.11). Interestingly, the reduction in GPR30 in SHR is in contrast to the increase in GRK2 expression. Whether this reduction in GPR30 expression is related to hypertension remains to be determined. Our initial experiments examining the expression levels in isolated tissue extracts or VSMCs protein lysates were performed with one of the only useable

commercially available antibody (MBL International) available against GPR30. Unfortunately, during the course of these experiments the company stopped producing the antibody. We were therefore unable to complete the assessment of GPR30 expression in younger (5 week old) normotensive WKY and SHR rats. I have tried many different antibodies from different companies without any luck of detecting GPR30 in tissue and/or cell extracts. I even examined lysates from cells in which we over-expressed our flag-tagged GPR30 (with adenovirus) and could detect GPR30 expression as assessed with the M2 (flag-tag) antibody but could not detect GPR30 expression using the many different commercially available GPR30 antibodies.

The loss of GPR30 expression in VSMCs in culture (Figure 3.12) has potentially significant implications. For both aldosterone and estradiol, the balance of their vasodilator vs. vasoconstrictor effects are reflective of the balance of their effects at endothelial vs. smooth muscle cells. Any enhancement of vascular smooth muscle mediated contractile effects, as might be seen with increased GPR30 expression, would be expected to tip the balance towards greater vasoconstrictor effects. Thus, dynamic regulation of GPR30 expression in VSMCs might be a potential mechanism regulating the overall contractile effects of both aldosterone and estrogens. Interestingly, this dynamic regulation of GPR30 expression in vascular smooth muscle cells is seen *in vivo* following vascular injury, while MR and ER receptor expression is maintained (R. Gros, Q. Ding, et al., unpublished observations). If this is a generalized phenomenon, then GPR30- mediated enhancement of aldosterone- and estradiol- stimulated contractile responses might be expected to be important in the

pathobiological responses to vascular injury and perhaps hypertension or other cardiovascular diseases.

In the current study, the potential effect of GPR30 activation on vascular reactivity was assessed, using an approach that had been previously used to delineate the vasodilator and/or vasoconstrictor effects of aldosterone in isolated aortic ring segments (Liu et al, 2003). Our previous study suggested that the effect of Aldo to attenuate vasoconstrictor responses was endothelial/NO dependent. First, the ability of Aldo to attenuate vasoconstrictor responses was lost after endothelial denudation. In fact, in the absence of the endothelium, the effect of Aldo was converted to a vasoconstrictor response, reminiscent of the pattern of response to acetylcholine in endothelium-intact versus denuded preparations (Touyz et al, 1994). Second, in rings from SHR, which demonstrate endothelial dysfunction (Konishi & Su, 1983), the ability of Aldo to attenuate vasoconstrictor responses was lost. Lastly, the effect of Aldo to attenuate vasoconstriction was lost after inhibition of NOS activity by L-NMMA (Liu et al, 2003). Based on these previous data, I examined the effects of aldosterone and G1 in isolated aortic ring segments. Both aldosterone and G1 were able to attenuate PE-mediated vasoconstriction in isolated endothelium-intact aortic ring segments (Figures 3.13 and 3.15). These studies confirmed our previous findings (Liu et al, 2003) regarding the ability of aldosterone to mediate vasodilation in endothelium- intact ring segments as well as implicating GPR30 as a potential mediator of vasodilation since G1 was also able to attenuate PE-mediated constriction. Therefore, I examined the effects of G15 on either aldosterone or G1-mediated

vasodilation, which has been suggested to be a GPR30-specific antagonist. Interestingly, only G15 pre-treatment was able to inhibit the vasodilatory effect of aldosterone (Figure 3.14), and not G1 (Figure 3.16). The reason for the discrepancy is not currently known, but warrants further investigation in the future perhaps with newer and different GPR30 agonists. Interestingly, I did observe that removal of the endothelium was associated with a reduction in G1-mediated vasodilation (Figure 3.17). This observation leads me to examine the effects of different GPR30 agonists (eg. aldosterone, G1) on cultured endothelial cells. Previous studies have suggested a potential role of GPR30 in regulating cellular growth and apoptosis (Gros et al, 2011b). Therefore, I utilized the wound healing assay to examine the effect of GPR30 activation on EC migration and proliferation. My initial studies examined the effects of estrogen, aldosterone and G1 on wound healing (Figure 3.18). Interestingly, only G1 had a significant effect on slowing wound healing as compared to estrogen or aldosterone, but this was not altered by pre-treatment with G15 (Figure 3.18). To further examine this effect, I performed dose-response experiments with G1 in the absence and presence of G15 (Figure 3.19). Surprisingly, wound healing of ECs was very sensitive to G1, within a 0.1-1 μM range, and again G15 did not block the effects of G1. Due to the inability of G15 to block the G1 response in endothelial cells, I utilized a SiRNA approach to knockdown GPR30 expression. Despite knocking down GPR30 expression in endothelial cells, G1 was still able to significantly inhibit wound healing (Figure 3.20). This inability of G15 to block G1 responses in either isolated ring segments or cultured endothelial cells or by knocking down GPR30 expression

suggests that G1 may act as an agonist on receptor(s) other than GPR30. Based on GPR30 knocking down and parallel experiments using VSMCs, this G1 effect on wound healing could be GPR30 independent and EC specific.

In total, we have studied the effect of GPR30 in both aortic rings and cultured vascular cells. Earlier data have shown that the expression of GPR30 is reduced in tissues from SHRs. One possible interpretation of the down-regulation of GPR30 in SHRs is elevated receptor internalization and degradation. In the current study, I examined the possibility that GRK2 is able to interact and/or phosphorylate GPR30 and regulate its desensitization, internalization and degradation. In double immunofluorescence assay, little or no co-localization between GPR30 and GRK2 was observed under basal or stimulated condition (Figure 3.22). Co-immunoprecipitation data also suggested that there was no direct interaction between GRK2 and GPR30 in cultured VSMCs (Figure 3.23). Explanation for these data could be: 1) there was no endogenous GPR30 expression in cultured VSMCs, or GPR30 over-expression using adenovirus infection could affect the binding between GPR30 and GRK2; 2) other GRKs or protein kinases could bind to GPR30 and be responsible to the desensitization of GPR30; 3) there is controversy about the localization of GPR30. It has been suggested that GPR30 is predominately localized to the endoplasmic reticulum (ER), and that only minor amounts of receptor are detectable at the cell surface (Cheng et al, 2011). If this is right, then it is unlikely that GRK2 could interact with GPR30 because GRK2 normally tends to phosphorylate receptors located on the plasma membrane.

4.3 Summary and conclusions

In summary, my studies have demonstrated that acute regulation of GRK2 expression levels may be important in regulating the function of vascular smooth muscle cells in culture. Overwhelming data has implicated GRKs in cardiovascular diseases including hypertension. This would suggest that a better understanding of the potential regulation of GRK2 (and other GRKs) and will therefore be an important target for future investigation. Whether this can be done in tissue and/or in vivo remains to be determined, but may require novel therapeutics (stem cells, small molecules etc.). In addition, I have demonstrated the potential role of the recently identified steroid receptor, GPR30 in mediating vascular reactivity as well as the potential reduced expression of this receptor in hypertensive animals. What role this reduced expression and/or altered signaling has in the hypertensive state remains to be determined. Cardiovascular diseases, such as hypertension are very prevalent and affect many Canadians. Therefore a better understanding of the regulation of vascular smooth muscle cells and endothelial cells under both physiological and pathological conditions remains an important focus.

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APPENDICES

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