Angiotensin II-induced Cyclooxygenase 2 Expression in Rat Aorta Vascular Smooth Muscle Cells Does Not Require Heterotrimeric G Protein Activation

Thomas A. Morinelli, Ryan T. Kendall, Louis M. Luttrell, Linda P. Walker and Michael E. Ullian

Divisions of Nephrology (TAM, LPW, MEU) and Endocrinology (RK, LL), Department of Medicine, Medical University of South Carolina, and the Ralph H. Johnson Veterans Administration Hospital (TAM, LML, MEU), Charleston, South Carolina
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b) Corresponding author:

Thomas A. Morinelli, Ph.D.
Division of Nephrology, Department of Medicine
829 Clinical Sciences Building
96 Jonathan Lucas Street
Charleston, SC 29425
843-792-0071 (OFFICE)
843-876-5129 (fax)
morinelt@musc.edu

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d) Abbreviations:

SII-AngII; Sarcosine1-Ile4-Ile8-Angiotensin II
AngII: Angiotensin II
LSCM: Laser Scanning Confocal Microscopy
RASMC: rat aorta smooth muscle cells
GPCR: G protein coupled receptor
COX-2: Cyclooxygenase 2
GFP: green fluorescent protein

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ABSTRACT

Angiotensin II (AngII) initiates cellular effects via its G protein-coupled AT1 receptor (AT1R). Previously, we showed that AngII-induced expression of the prostanoid-producing enzyme cyclooxygenase 2 (COX-2) was dependent upon nuclear trafficking of activated AT1R. In the present study, mastoparan (an activator of G proteins), suramin (an inhibitor of G proteins), U73122 (a specific inhibitor of phospholipase C) and Sarcosine-Ile⁴-Ile⁸-Ang II (SII-AngII, a G protein-independent AT1R agonist) were used to determine the involvement of G proteins and AT₁AR trafficking in AngII-stimulated COX-2 protein expression in HEK-293 cells stably expressing AT₁A/GFP receptors and cultured vascular smooth muscle cells, respectively. Mastoparan alone stimulated release of intracellular calcium and increased COX-2 expression. Pre-incubation with mastoparan inhibited AngII-induced calcium signaling without altering AngII-induced AT₁A trafficking, p42/44 ERK activation or COX-2 expression. Suramin or U73122 had no significant effect on their own, did not inhibit AngII-induced AT₁A trafficking, p42/44 ERK activation or COX-2 expression, but did inhibit AngII-induced calcium responses. SII-AngII stimulated AT₁A trafficking and increased COX-2 protein expression without activating intracellular calcium release. These data suggest that G protein activation results in increased COX-2 protein expression, but AngII-induced COX-2 expression appears to occur independently of G protein activation.
INTRODUCTION

Angiotensin II (AngII), the vasoactive hormone of the renin-angiotensin system, initiates its cellular effects through activation of its cognate seven transmembrane-spanning G protein-coupled receptor, angiotensin AT_{1A} receptor (AT_{1AR}). Interaction of AngII with the AT_{1AR} initiates conformational changes in the receptor, producing activation of its targeted G protein, G_{q/11}. Subsequent to receptor-mediated activation of G_{q/11}, a cascade of intracellular signaling events occurs, including acute activation of phospholipase C, release of intracellular stores of calcium and subsequent activation of numerous kinases including p42/44 ERK (Millan et al., 1991; de Gasparo et al., 2000) and chronic cellular effects including induced protein synthesis of renin, angiotensinogen and cyclooxygenase 2 (COX-2) (Eggena et al., 1993; Ohnaka et al., 2000; Morinelli et al., 2008).

Subsequent to G protein activation, GPCRs undergo β-arrestin-mediated internalization. In addition to serving as a means to interrupt cell signaling from the cell surface, receptor internalization also serves to continue the signaling cascade within the cell. This process, in which internalized endosomes containing de-sensitized receptors along with attached β-arrestins act as a scaffold, interacting with specific signaling proteins such as p42/44 ERK, has been described as a signalsome (Luttrell et al., 2001). These “signalsomes’ are responsible for the prolonged activation of p42/44 ERK by GPCR agonists such as AngII.

In addition to the above-mentioned role of receptor trafficking in desensitization/recycling and cytoplasmic signaling, an additional pathway for GPCR trafficking has been proposed, namely localization of the activated receptor to the nuclear area. (Re et al., 1983; Eggena et al., 1993). Lu et al demonstrated nuclear translocation of the AT_{1AR} in response to cellular activation by AngII (Lu et al., 1998). We have previously shown that nuclear localization of the receptor
may be dependent upon a putative nuclear localization sequence within the carboxy tail and that localization to the nuclear area from the plasma membrane involves clathrin-coated pits and is associated with the ability of AngII to induce COX-2 protein expression (Morinelli et al., 2007; Morinelli et al., 2008).

The generation of prostanoids, via the activation of cyclooxygenase (COX-1 and/or COX-2), is responsible for a plethora of physiological and pathological responses. The activity of constitutive COX-1 results in the generation of prostanoids utilized to maintain physiologic homeostasis. In rat aorta vascular smooth muscle cells (RASMC), AngII induces the transcription for COX-2 via involvement of nuclear factor-κB (NF-κB) and mediation of several cytoplasmic kinases including Pyk2, MEKK4 and p38 (Ohnaka et al., 2000; Hu et al., 2002; Derbyshire et al., 2005). Thus, AngII has been implicated in the regulation of COX-2 and the activation of several chronic disease processes mediated by COX-2.

The present study was designed to elucidate further the pathway for nuclear localization of the AT1AR by testing the hypothesis that AngII-induced COX-2 expression in RASMC is not dependent upon the mediation of G proteins, in particular Gq. To test this hypothesis, we utilized mastoparan (an activator of G proteins), suramin (an inhibitor of G protein activation), U73122 (a specific inhibitor of phospholipase C [PLC] activation) and Sarcosine\(^1\), Ile\(^4\), Ile\(^8\)-AngII (SII-AngII) an AT1R agonist whose activation of cellular responses has been shown to be independent of G proteins (Wei et al., 2003).
METHODS

**Cell culture:** Primary culture of rat aorta vascular smooth muscle cells (RASMC) was performed as previously described (Morinelli et al., 2008). Cells were maintained in Dulbecco’s modified Eagle’s Medium (DMEM) high glucose supplemented with 10% fetal bovine serum (FBS) and 1% antibiotic/antimycotic/ fungizone and used between passages 3 through 8. HEK-293 cells (American Type Culture Collection) stably expressing a wild-type AT\textsubscript{1}AR/Green Fluorescent Protein (AT\textsubscript{1}AR/GFP) construct were maintained using Ham’s F12 media supplemented with 10% FBS, 1% antibiotic/antimycotic/fungizone and G418 (400 µg/ml) (Morinelli et al., 2007). Cell culture media and supplements were obtained from GIBCO-BRL (Grand Island, NY).

**Radioligand binding assays:** Binding studies employing \[^{125}\text{I}\]-AngII were performed as previously described (Morinelli et al., 2008). Confluent monolayers of RASMC in 6 well plates were exposed to the various compounds for 30 min. at 37°C. Subsequently, the growth medium containing the compounds was removed and binding buffer containing \[^{125}\text{I}\]-AngII (~200,000 cpm, ~100 fmoles) ± the AT1R antagonist losartan (10 µM) was added to the cells and incubation was carried out at 4°C for 90 min. Subsequently, cells were washed with cold saline buffer to remove unbound radioligand and then solubilized in 0.1% sodium dodecyl sulfate (SDS)/0.1 M NaOH and associated radioactivity counted. Specific binding was determined, i.e. the difference between radioactivity associated with the cell lysates in the absence and presence of losartan, and corrected for total cell protein per well.

**Laser scanning confocal imaging:** HEK-293 cells stably expressing the wild-type AT\textsubscript{1}AR/GFP construct were plated onto collagen-coated 25-mm glass cover slips in 6-well plates and maintained in selection medium. Prior to study, cells were deprived of serum (0.1% FBS) for 24 – 48 hours. On the day of study, compounds were added directly to the media and incubated for
30 min. followed by addition of angiotensin II (100 nM) for 60 min. and fixed with 4% paraformaldehyde solution in PBS for 15 minutes at room temperature. Cells were washed 2x with PBS followed by addition of the DNA fluorescent dye DRAQ5 (2 µM, Alexis Corp., San Diego, CA). Confocal microscopy was performed using a Zeiss LSM 510 META laser-scanning microscope (Carl Zeiss, Inc.) equipped with a 60x objective, using the following laser wavelengths: GFP, excitation 488 nm, emission 505-530 nm and for DRAQ5 excitation 543 nm, emission 560-615 nm.

**Calcium Measurements:** RASMC were plated into 96 well clear-bottom black plates at a density of 60,000 cells/well. The next day, media was changed to 0.1% bovine serum albumin (BSA)/DMEM. Twenty-four to Forty-eight hours later, media was removed and cells were incubated with the calcium sensitive fluorescent probe Fluo-3AM (2 µM; Molecular Probes) in Hanks Balanced Salt Solution (HBSS) pH 7.4, containing 2.5 mM Probenecid and 0.1% bovine serum albumin for 60 minutes, 37°C. At the end of the incubation, the cells were washed 3 times with HBSS and placed into a Fluorometric Imaging Plate Reader (FLIPR, Molecular Devices) and exposed to the various compounds for 30 min., followed by AngII. Increases in intracellular free calcium (Cai) were reflected by increases in detected fluorescence.

**Immunoblotting:** Confluent monolayers of RASMC were serum-deprived (0.1% FBS) for 24 – 48 hours. Cells, in cell culture media (COX-2 expression studies) or HBSS, 20 mM HEPES, pH 7.4 (p42/44 ERK activation assays) were exposed to vehicle, mastoparan (10 μM, Biomol International), suramin (10 μM, Tocris Bioscience), or U73122 (10 μM, Calbiochem) for 30 minutes 37°C, followed by addition of AngII (Sigma-Aldrich). At the end of the incubation period with AngII, the cells were washed 2x with ice-cold PBS followed by addition of radioimmunoprecipitation assay buffer (RIPA buffer) (50 mM Tris-HCl, pH 7.4, 150 mM NaCl,
1 mM EDTA, 1% NP-40, 0.25% Na-deoxycholate plus protease inhibitor cocktail I; 500 mM AEBSF, 150 nM aprotinin, 1 mM E-64 and 1 mM leupeptin, Calbiochem) for p42/44 ERK assays. For COX-2 assays, cells were lysed directly with 1x SDS/PAGE buffer. Lysates (10-20 µg) were separated by SDS-PAGE (4-20% gradient), transferred to nitrocellulose and probed for the presence of phosphorylated p42/44 ERK (phospho-p42/44) and total p42/44 ERK (1:2000, Cell Signaling Technology, Beverly, MA) according to manufacturers’ directions. Blots were stripped of antibodies between probing for phosphorylated and total proteins. In COX-2 expression studies, the COX-2 protein was detected using a rabbit polyclonal antibody (1:200, Upstate, Lake Placid, NY). Protein loading and transfer was corrected for by detection of β-Actin (1:5000, Sigma-Aldrich, St. Louis, MO). Detection of protein bands was performed by addition of CDP-Star reagent (New England Biolabs, Ipswich, MA) and visualized by exposure of the nitrocellulose to radiographic film (X-OMAT, Kodak). Quantitation of the visualized protein bands was performed by densitometric scanning of the exposed radiographic film (Kodak Molecular Imaging Software, Rochester, NY). The density ratio for phosphorylated protein to total protein was used as an indicator of kinase activation.

Statistics: Values shown are Means ± SE from the indicated number of studies (n) and comparisons were made using Microsoft Excel data analysis package employing ANOVA with Fisher’s post hoc t-test or Kruskal-Wallis with Mann-Whitney test where indicated. Significance was tested at the 95% level.
RESULTS

Activation of heterotrimeric G proteins, in particular Gq/11 and G12/13, by the AT1AR mediate cellular responses to AngII. Small molecular compounds such as suramin or naturally occurring peptides such as the wasp venom peptide mastoparan have been used to probe the involvement of G protein activation in cell signaling events. Suramin has been demonstrated to have multiple effects on G proteins, including the interruption of G protein/receptor interaction by blocking GDP to GTP exchange, and to promote epithelial cell proliferation. Mastoparan, conversely, has been shown to activate G protein signaling by promoting GDP to GTP exchange preferentially with G_i and G_o, resulting in numerous cellular effects including enhanced GTPase activity, increased activation of phospholipase D activity and AT1AR desensitization. AT1AR-induced activation of phospholipase C (PLC) with the resultant release of inositol 1,4,5-trisphosphate (IP3) from membrane phospholipids results in release of calcium from intracellular storage sites. This activation of PLC is mediated by AT1AR activation of G_q. A pharmacological agent commonly used to demonstrate a role of G_q activation of PLC is the compound U73122, which inhibits PLC-dependent processes.

We first examined the ability of these compounds to alter the interaction of AngII with its cell-surface receptor. Pre-treatment of monolayers of RASMC with mastoparan, suramin or U73122 had no significant effect on AngII binding to cell surface AT1ARs as detected by specific binding of [125I]-AngII (Figure 1).

In order to determine if these compounds alter basal plasma membrane expression of AT1AR or AngII-induced internalization and intracellular trafficking AT1AR, AT1AR/GFP/HEK cells were examined by confocal microscopy after treatment with mastoparan, suramin or U73122, followed by exposure to AngII (Figure 2). This cell line has been previously characterized and is

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a useful cell model to visualize AT$_{1a}$R trafficking (Morinelli et al., 2007). Addition of AngII to untreated cells produced characteristic localization of the receptor to the nuclear membrane area which has been observed previously (Morinelli et al., 2007). Exposure of cells to mastoparan, suramin or U73122 alone did not alter basal distribution of the receptor; ie AT$_{1a}$R/GFP-expressing cells maintained a plasma membrane distribution of the receptor similar to untreated cells. Subsequent addition of AngII to these cells produced the characteristic AT$_{1a}$R internalization and nuclear membrane localization.

One of the earliest events in the signaling cascade initiated by the AT$_{1a}$R is $G_q$-mediated activation of PLC with resultant increase in Ca$_i$. RASMC were treated with mastoparan, suramin or U73122 prior to exposure to AngII (Figure 3). Pre-treatment with mastoparan caused an elevation in Ca$_i$, suggesting that mastoparan directly activated $G_q$ and subsequently PLC, producing a release of calcium from intracellular stores. Addition of suramin or U73122 had no effect on Ca$_i$, as expected. Mastoparan treatment, subsequent to producing a direct effect on calcium, blocked the ability of AngII to increase Ca$_i$. Pre-treatment with suramin also inhibited the ability of AngII to elevate Ca$_i$. U73221, an inhibitor of PLC, as expected, also blocked AngII-induced elevations of Ca$_i$. These results confirm the role of G proteins in AngII-induced elevations of Ca$_i$.

G protein-mediated activation of the mitogen-activated kinase (MAPK) pathway is a central element to the hypertrophy/hyperplasia response of many GPCRs. One of the key intermediary kinases in this pathway is the serine/threonine kinase p42/44 ERK. RASMC, when exposed to AngII, produce a rapid and reversible phosphorylation of p42/44 ERK. We examined the ability of mastoparan, suramin and U73122 to influence the ability of AngII to activate this kinase. Mastoparan, an activator of heterotrimeric G proteins, activated p42/44 ERK (Figure 4).
effect appeared to be additive to the stimulation produced by AngII. Suramin, a reported inhibitor of heterotrimeric G proteins, had no direct effects on p42/44 ERK and did not block the stimulation produced by AngII. U73122, an inhibitor of PLC, appeared to produce a small but not significant stimulatory effect on p42/44 ERK without effecting AngII-induced activation. These results support the concept that AngII-induced activation of MAPK signaling can occur by both G protein-dependent and independent pathways.

Having seen the varied effects of G protein interacting compounds on AngII-activated intracellular signaling, we next examined their effects on AngII-induced COX-2 expression. RASMC were pre-treated with vehicle, mastoparan, suramin or U73122 for 30 minutes, followed by addition of buffer or AngII for an additional 3 hours, and expression of COX-2 was determined (Figure 5). As seen previously, AngII increased COX-2 protein expression in a concentration-dependent manner reaching approximately a 3-fold increase at 100 nM. Mastoparan, an activator of G proteins, which produces elevations of Ca_i and activation of p42/44 ERK, also produced significant increases in the expression of COX-2. When AngII was added to mastoparan pre-treated RASMC, COX-2 expression was further increased. The G protein inhibitor suramin, which does not alter AT_1AR trafficking or p42/44 ERK activation but inhibits AngII-induced Ca_i, had no effect on COX-2 expression on its own and suppressed slightly but not significantly the ability of AngII to increase COX-2 expression. U73122, an inhibitor of PLC, which does not alter AT_1AR trafficking but inhibits elevations in AngII-induced Ca_i, did not inhibit AngII-induced COX-2 protein expression.

The effects seen with mastoparan, suramin and U73122 suggest that AngII-induced COX-2 expression depends partially on G protein activation and partially on AT_1AR intracellular (nuclear) trafficking.
Since these data suggest that AngII activation of AT$_1$ARs and subsequent COX-2 protein expression may not require the mediation of G proteins, we examined the ability of the G protein-independent AT$_1$AR agonist SII-AngII to activate the receptor and induce expression of COX-2. SII-AngII is a ligand of the AT$_1$AR that can induce receptor internalization and p42/44 ERK activation independent of G protein activation (Wei et al., 2003). Cells exposed to SII-AngII produced endocytosis of the AT$_1$AR/GFP construct, which was inhibited by the AT1R antagonist losartan, and increased expression of the COX-2 protein without eliciting elevations in intracellular free calcium (Figure 6).
DISCUSSION

Our previous studies suggest that AngII-mediated increases in COX-2 protein expression depend on nuclear membrane localization of activated AT_{1A}R subsequent to internalization through clathrin-coated pits (Morinelli et al., 2007; Morinelli et al., 2008). The present study investigated the role of heterotrimeric G proteins in this process, with data summarized in Table 1. Mastoparan, an activator of heterotrimeric G proteins, increased Ca_{i}, blocked AngII-stimulated increases in Ca_{i}, activated p42/44 ERK, increased the expression of COX-2 and enhanced AngII-induced expression of COX-2, without altering nuclear membrane trafficking of AT_{1A}Rs. Suramin, an inhibitor of heterotrimeric G proteins, inhibited AngII-induced Ca_{i} but had no effect on surface expression or nuclear membrane localization of AT_{1A}Rs and had no significant effect on AngII-induced COX-2 expression. U73122, an inhibitor of G_{q}-dependent PLC activation, inhibited AngII-induced Ca_{i} and activation of p42/44ERK but had no inhibitory effects on AT_{1A}R surface expression, nuclear membrane receptor localization or AngII-induced COX-2 expression. SII-AngII, a heterotrimeric G protein-independent activator of AT_{1A}R-signal transduction pathways, promoted nuclear membrane trafficking of AT_{1A}Rs and increased the expression of COX-2 protein without elevating intracellular free calcium, a G_{q}-dependent event.

The ability of cells to respond to AngII relies on the interaction of AngII with its cell surface G protein-coupled receptor. The major class of AngII receptors is the AT1 receptor, with the AT_{1A} receptor being the subtype found in vascular smooth muscle cells. Activation of this receptor by AngII produces well-characterized cellular effects related to its physiological/pathological activities, including cell contraction, protein synthesis and cell proliferation. The G proteins G_{q/11} mediate the majority of these cellular effects. Additional evidence suggests that AT1R under select conditions may also activate other G proteins such as
G_{12/13} and possibly G_{i/o} (de Gasparo et al., 2000). Coincident with the activation of the above
mentioned signaling cascade, the activated AT_{1A}R, as with other G protein-coupled receptors
(GPCRs), initiates a signal-termination sequence producing activation of specific G protein
receptor kinases (GRKs), which produce phosphorylation of the receptor resulting in \( \beta \)-arrestin-
mediated receptor desensitization, halting further activation of G proteins and directing the
receptor into clathrin-coated pits along the surface of the membrane. The internalized pits are
targeted to acidic endosomes (lysosomes), where the receptor complex is either dissociated as a
result of de-phosphorylation of the receptor produced by the acidic environment and rapidly
recycled back to the plasma membrane (Class A GPCRs) or, for Class B GPCRs, held in the
endosomes, with \( \beta \)-arrestin attached (Oakley et al., 2001; Luttrell and Lefkowitz, 2002).

More recent evidence points to an additional pathway for cellular responses to AngII
involving the epidermal growth factor receptor (EGFR) (Shah and Catt, 2006). In this paradigm,
activation of AT_{1}R results in metalloproteinase-dependent release of surface bound epidermal
growth factor and subsequent activation of the EGFR followed by activation of its signaling
pathway. Alternatively, non-G protein-dependent signaling pathways for AngII have been
recently described in which the internalization of the receptor initiated by \( \beta \)-arrestin interaction
permits subsequent prolonged activation of the MAP kinase pathway as a result of interaction of
the kinases with the receptor/\( \beta \)-arrestin scaffold (Pierce et al., 2000).

In order to examine the involvement of heterotrimeric G protein activation in AngII
stimulated COX-2 protein expression, we used small, cell-permeable compounds that either
stimulate or inhibit G proteins. Mastoparan, a peptide derived from wasp venom, activates G
proteins by promoting the exchange of GTP for GDP, thus mimicking activation of G proteins by
GPCR agonists (Higashijima et al., 1988). This tetradecapeptide has been shown to regulate
numerous G protein signaling events, including Ca\(^{2+}\)-ATPases, the monomeric G proteins rho and rac and phospholipase D (Jones and Howl, 2006). To identify regions of the AT\(_1\)AR involved in receptor desensitization, Tang et al used mastoparan to desensitize the AT\(_1\)AR. Treatment of Chinese hamster ovary (CHO) cells expressing AT\(_1\)AR with mastoparan resulted in desensitization of PLC to subsequent addition of AngII. The mastoparan-induced desensitization of the AngII response was comparable to that seen for pre-treatment with AngII itself, ie homologous desensitization (Tang et al., 1998). In our present studies, mastoparan also produced a densitization of the AngII-induced calcium response (a G\(_q\)-PLC dependent signal) but did not inhibit AngII-induced AT\(_1\)AR trafficking and COX-2 protein expression. Mastoparan, on its own, did produce an increase in intracellular free calcium, activate p42/44 ERK and also increased COX-2 protein expression. This supports published data showing G protein activation of PLC and the p42/44 ERK pathway and also implicates this pathway in COX-2 protein expression.

Another technique for exploring the role of G proteins in the AngII-induced expression of COX-2 protein is to utilize compounds that have been shown to inhibit the activity of G proteins. A family of small compounds that inhibit G proteins and thus inhibit the effects of GPCRs has been developed from the original suramin. Suramin inhibits G protein activity by interfering with the association of the G\(_\alpha\) and G\(_{\beta\gamma}\) subunits, thus blocking the G protein-signaling pathway (Beindl et al., 1996; Freissmuth et al., 1996). The ability of suramin to block G protein-dependent signaling pathways has led to many studies documenting its anti-cancer effects. However, recent evidence suggests that suramin, in certain cell types such as CHO cells and renal epithelial cells, may actually activate signaling pathways related to cell proliferation (Nakata, 2004; Zhuang and Schnellmann, 2005). In the present study, treatment of cells with
suramin did not alter surface expression of AT1ARs, AngII-induced AT1AR trafficking, p42/44 ERK activation or COX-2 expression. However, suramin did inhibit Gq-dependent PLC activation and increase of intracellular free calcium. In a likewise manner, U73122, an inhibitor of PLC activity, did not alter any of the responses examined in this study except that for AngII-induced elevations of intracellular free calcium. These data indicate, as discussed above, that activation of G proteins by AT1AR is not essential for AngII to initiate nuclear membrane localization of its receptor and subsequently increase COX-2 expression. Additionally, these data show that elevation of Ca_i does not necessarily mediate changes in COX-2 protein expression.

SII-AngII is a selective agonist for the AT1AR, in that this ligand can activate β-arrestin-mediated AT1AR internalization and p42/44 ERK activation without G protein activation. In our studies, use of SII-AngII promoted internalization and nuclear membrane localization of the AT1AR and also increased COX-2 protein expression in the absence of increases in intracellular free calcium, thus confirming lack of G protein activation in its signaling.

In summary, the present study examined the role of G protein activation in AngII-induced expression of the enzyme COX-2. Through the use of compounds that stimulate G proteins, inhibit G proteins or inhibit an enzyme activated by a G protein, we conclude that the ability of AngII to increase COX-2 expression was dependent upon normal internalization and nuclear membrane trafficking of the AT1AR. G protein-dependent activation of PLC and subsequent elevations in intracellular free calcium is not required for this effect, since suramin or U73122 did not alter AT1AR trafficking and did not inhibit AngII-induced COX-2 expression. Previous studies implicated activation of the MAP kinase pathway in AngII-induced COX-2 expression (Ohnaka et al., 2000). The MAP kinase pathway may be a parallel pathway for activation of
COX-2 expression, since mastoparan activated p42/44 ERK and also increased COX-2 expression. Lastly, the ability of the selective AT1AR agonist SII-AngII to promote AT1AR internalization, nuclear membrane localization and COX-2 protein expression without causing an elevation in intracellular free calcium, a G protein-PLC dependent event, again supports the concept that G protein activation is not a requirement for AngII to increase the expression of COX-2, whereas receptor internalization and nuclear localization may be required.
REFERENCES


FOOTNOTES:

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Reprint Requests:

Thomas A. Morinelli, Ph.D.
Division of Nephrology, Department of Medicine
829 Clinical Sciences Building
96 Jonathan Lucas Street
Charleston, SC 29425
Email: morinelt@musc.edu
LEGENDS FOR FIGURES

Figure 1. Effect of G protein-interacting compounds on Ang II binding. RASMC were treated with vehicle (Untreated), mastoparan (10 µM), suramin (10µM) or with U73122 (10 µM) for 30min, 37°C. Media was removed and [125I]-angiotensin II radioligand binding was determined as described. Specific radioactivity associated with the cells was determined and corrected for total cell protein. Values shown are the average (± sem) from 3 studies.

Figure 2. Effect of G protein-interacting compounds on AngII-induced AT_{1A}R intracellular trafficking. Representative LSCM images from HEK-293 cells stably expressing AT_{1A}R/GFP exposed to vehicle (Untreated), mastoparan (10 µM), suramin (10µM), or U73122 (10 µM), 37°C, 30 min followed by stimulation with AngII (100 nM, 60’). Cells were fixed and prepared for imaging as described. AT_{1A}R/GFP is seen as green while nuclei are visualized with the DNA specific dye DRAQ 5 and seen as red. Corresponding DIC images also shown.

Figure 3. Effect of G protein-interacting compounds on AngII-induced calcium elevations. A) RASMC were exposed to vehicle (Untreated), mastoparan (10 µM), suramin (10µM), or U73122 (10 µM), indicated by arrow at 0 min, and monitored for changes in fluorescence as a measure of elevations in intracellular free calcium as described. Subsequently, these same cells were exposed to AngII (100nM), indicated by arrow at approximately 22 min, and monitored for changes in fluorescence. (RFU; relative fluorescence units). B) Summary of maximum changes in intracellular fluorescence (C_{a_i}) in response to AngII following pre-exposure to the indicated compounds (average values ± SEM, n=6). * p < 0.05 vs. Untreated (ANOVA with Fisher’s post-hoc test).
Figure 4. Effect of G protein-interacting compounds on AngII-induced activation of p42/44 ERK. A) Representative immunoblots from lysates of RASMC exposed to vehicle (Untr), mastoparan (MAST, 10 µM), suramin (SUR, 10µM), or U73122 (U73, 10 µM), 37°C, 30 min followed by stimulation with AngII (100nM, 5'). Detection of activated, phosphorylated (p) p42/44 ERK was performed as described. Blot was stripped and re-probed with antibody for total p42/44 ERK as described. B) Summary of densitometric scanning of immunoblots for detection of phospho-p42/44 ERK in response to stimulation by AngII following pre-exposure to the indicated compounds (average values ± SEM, n=3-5, * p < 0.05 vs. non-stimulated (-); + p < 0.05 vs. non-stimulated, untreated (-).

Figure 5. Effect of G protein-interacting compounds on AngII-induced expression of COX-2. RASMC were pre-exposed to vehicle (Control), mastoparan (10µM), suramin (10µM) or U73122 (10µM). Thirty minutes later, cells were exposed to vehicle (0) or indicated concentrations of AngII for three hours followed by cell lysis and immunoblotting to detect for the expression of COX-2. A) Representative immunoblot showing increased expression of COX-2 after AngII treatment and effects of various compounds on this increased expression. Detection of β-actin used to correct for protein loading. B) Summary of densitometric scanning of immunoblots showing average values ± SEM, n = 3-9. * p < 0.05 vs. unstimulated (0), Control cells; † p < 0.05 vs. unstimulated cells (0).

Figure 6. Effects of SII-AngII on AT1aR signaling and COX-2 protein expression. A) AT1aR/GFP internalization. LSCM imaging of HEK-293 cells stably expressing AT1aR/GFP
were exposed to vehicle (i), SII-AngII (5 µM, (ii)), or losartan (10 µM) then SII-AngII (iii) for 60’ at 37°C. Cells were fixed with formaldehyde as described and prepared for imaging. The specific DNA dye DRAQ5 was used to visualize nuclei. Arrows indicate nuclear localized receptors. Scale bar is 5 um. Representative images from 2 similar studies. B) Intracellular calcium response. AT1A R/GFP/HEK cells were loaded with the calcium sensitive dye fluo-3AM and changes in intracellular calcium determined as described. AngII (100 nM) or SII-AngII (5 µM) was added where indicated (arrow). C) Immunoblot of COX-2 expression from lysates of RASMC exposed to various concentrations of SII-AngII or AngII (100nM) for 3 hours. Cell lysates prepared and detection of COX-2 was determined as described. D) Summary of densitometric analysis of SII-AngII induced COX-2 expression in RASMC. Mean values ± SEM from 3 similar studies.
Table 1. Summary of effects of G protein interacting compounds on AngII receptor binding and cell signaling. ND; Not Determined

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<td>ND</td>
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</table>

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Figure 2

Untreated vs. AngII

Mastoparan (10 μM) vs. Mastoparan (10 μM)/AngII

Suramin (10 μM) vs. Suramin (10 μM)/AngII

U73122 (10 μM) vs. U73122 (10 μM)/AngII
Figure 3

A.

- Untreated
- Mastoparan
- Suramin
- U73122

AngII

RFU

Time (min)

B.

Cai Response (%AngII)

Untreated  Mastoparan  Suramin  U73221

*
Figure 4

A.

<table>
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<tr>
<th>Treatment</th>
<th>Untr</th>
<th>U731</th>
<th>Sur</th>
<th>Mas</th>
<th>AngII</th>
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<tr>
<td>(-)</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td>(+)</td>
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**p-p42/44 ERK**

**p42/44 ERK**

B.

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<th>Treatment</th>
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<th>MAST</th>
<th>SUR</th>
<th>U731</th>
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<td>p-p42/44 ERK (fold increase)</td>
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* *
Figure 5

A.

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<td>-8</td>
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</tr>
<tr>
<td>β-Actin</td>
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<table>
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<th>U73122</th>
<th>AngII (M)</th>
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B.

![Bar chart showing COX-2 expression levels with different AngII concentrations and treatments]