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Angiotensin II Inhibits P-glycoprotein in Intestinal Epithelial Cells

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Abstract

Aim: P-glycoprotein (P-gp/MDR1) plays a major role in intestinal homeostasis. Decrease in Pgp function and expression has been implicated in the pathogenesis of IBD. However, inhibitory mechanisms involved in the decrease of Pgp in inflammation are not fully understood. Angiotensin II (Ang II), a peptide hormone predominantly expressed in the epithelial cells of the crypt-villus junction of the intestine has been shown to exert pro-inflammatory effects in the gut. It is increased in IBD patients and animals with experimental colitis. Whether, Ang II directly influences Pgp is not known.

Methods: Pgp activity was measured as verapamil-sensitive ³H-Digoxin flux. Pgp surface expression and exocytosis was measured by cell surface biotinylation studies. Signaling pathways were elucidated by western blot analysis and pharmacological approaches.

Results: Ang II (10 nM) significantly inhibited Pgp activity at 60 min. Ang II-mediated effects on Pgp function were receptor-mediated as the Ang II receptor 1 (ATR1) antagonist, Losartan blocked Pgp inhibition. Ang II effects on Pgp activity appeared to be mediated via PI3 kinase, p38 MAPK & Akt signaling. Ang II-mediated inhibition of Pgp activity was associated with a decrease in the surface membrane expression of Pgp protein via decreased exocytosis and was found to be dependent on the Akt pathway. Short-term treatment of Ang II (2 mg/kg b.wt., 2h) to mice also decreased the membrane expression of Pgp protein levels in ileum and colon.

Conclusion: Our findings provide novel insights into the role of Ang II and ATR1 in decreasing Pgp expression in intestinal inflammation.

Keywords

Intestine; Ang II receptor 1 (ATR1); MDR1; Akt; p38 MAPK; PI3K

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CONFLICT OF INTERESTS

The authors have no conflict of interests to declare

INTRODUCTION

P-glycoprotein (P-gp) is the functional product of the multidrug resistance gene 1 (MDR1). It is a glycosylated transmembrane protein expressed on the apical surface of epithelial cells in the small intestine and colon^{1,2}. Pgp contributes to the barrier function of the intestine by mediating the efflux of drugs/xenobiotics and bacterial toxins from the mucosa to the gut lumen^{1,3,4}. Moreover, the physiological role of Pgp/MDR1 was evident from studies in *mdr1* knockout mice showing that mice lacking *mdr1* gene develop spontaneous colitis similar to the human ulcerative colitis (UC)⁵. MDR1 gene has been shown to be located on chromosome 7 that also harbors the IBD (Inflammatory Bowel Diseases) susceptibility loci⁶. Patients with active UC and Crohn's disease (CD) showed a decrease in Pgp expression in the ileum and colon^{6,7}. This decrease in Pgp expression was also observed in experimental mouse models of intestinal inflammation e.g. dextran sulfate sodium (DSS) induced colitis⁸, IL10 knockout⁹ and TCR α (T cell receptor) knockout¹⁰ mice. These observations further imply that loss of intestinal epithelial integrity due to a non-functional Pgp appears to be a critical factor in the development of colitis. However, the mechanisms underlying the decrease in Pgp function and expression are not well known. In this regard, the renin-angiotensin system (RAS) and its key mediator Ang II have been shown to be involved in the development of inflammation in the gut¹¹ and play an important role in the pathogenesis of IBD in humans^{11,12}. In the renin-angiotensin system (RAS), Ang II is produced by the angiotensin I converting enzyme (ACE) from angiotensin I (Ang I), which is generated by renin from a precursor protein called angiotensinogen¹¹. Evidence suggests that Ang II peptide acts as a local cytokine in several organs systems involved in the regulation of inflammation and fibrosis¹¹. Reduced serum concentrations of ACE and increased colonic mucosal levels of Ang II in patients with Crohn's disease and ulcerative colitis have been reported^{12–14}.

Ang II is also capable of inducing production of reactive oxygen species (ROS) and increases the expression of pro-inflammatory cytokines such as IL-6 and TNF α in vascular smooth muscle cells^{11,15}. Ang II has been shown to induce pro-inflammatory effects in both *in vitro* cell culture and *in vivo* murine models. For example, Ang II increased prostaglandin synthesis via the stimulation of COX-2 expression in intestinal epithelial IEC-18 cells¹⁶. In the mouse colon, Ang II was also shown to induce the expression of inflammatory cytokines (CXC chemokines), generation of ROS and migration of neutrophils¹⁷. Ang II has also been shown to be involved in the pathogenesis of both TNBS (2,4,6 trinitrobenzene sulphonic acid)- and DSS-induced colitis^{18,19}.

Ang II mediates its actions via two membrane-bound G-protein coupled receptors, named the Ang II type 1 receptor subtype 1 (ATR1) and the subtype 2 (ATR2)^{11,15}. Both ATR1 and ATR2 have been shown to be present in the human small intestine, colon and intestinal epithelial Caco2 cells^{15,20}. Recent studies have shown that ATR1 plays an important role in the pathogenesis of intestinal inflammation¹⁹, whereas ATR2 appears to be 'tissue-protective', balancing the actions induced by activation of ATR1 in various pathophysiological conditions²¹, thereby, suggesting that ATR2 exerts actions opposite to ATR1. Previous studies demonstrated that specific ATR1 antagonist, CV-11974 (candesartan) protected against indomethacin-mediated intestinal injury in rats by decreasing

neutrophil infiltration into the intestinal mucosa²². Moreover, the increase in TNF α levels, neutrophil infiltration (source of ROS) and lipid peroxidation in the colonic mucosa were significantly ameliorated in (DSS)-treated ATR1_a-deficient mice¹⁹ suggesting the potential role of Ang II/ATR1 in colonic inflammation. Since decrease in Pgp plays an important role in the pathophysiology of intestinal inflammation, it is possible, therefore, that increase in Ang II contributes to the reduction in Pgp function and expression causing perturbed epithelial integrity. Therefore, the present studies were aimed at examining the direct effects of the pro-inflammatory peptide, Ang II on Pgp function and expression in intestinal epithelial cells in both in vitro cell culture and in vivo mouse models and delineate the mechanisms involved.

Our studies showed that Ang II under short-term conditions decreased Pgp function in Caco2 cells via the Ang II receptor 1 (ATR1) and signal transduction pathway involving PI3 kinase, p38 MAP kinase and Akt. Ang II-mediated decrease in the membrane levels of Pgp protein was found to be dependent on the Akt pathway. Furthermore, Pgp surface expression in Caco2 cells was reduced by Ang II via a decrease in Pgp exocytosis. In vivo administration of Ang II to mice showed a decrease in the membrane levels of Pgp protein in both ileum and colon. Our results, for the first time demonstrate that Ang II inhibits Pgp function and membrane expression via a post-translational mechanism.

RESULTS

Time course and dose response effects of Ang II on Pgp function in Caco-2 cells:

Caco-2 monolayers were treated with Ang II from the basolateral side for 15–60 min at 10 nM concentration or at different doses ranging from 0.1–10 nM (60 min) and Pgp function was measured as verapamil sensitive ³H-digoxin flux. As shown in Figure 1A, verapamil sensitive ³H-digoxin flux was significantly decreased (60%) at 60 min but not at 15 and 30 min time points compared to untreated control. Ang II did not show any effect at 0.1 nM concentration but significantly decreased Pgp activity at 1.0 nM & 10 nM concentrations. However, this decrease was more pronounced at 10 nM (Figure 1B). Thus, all the subsequent studies were performed using 10 nM concentration of Ang II. These data indicate that Pgp activity is inhibited by short-term treatment with Ang II in human intestinal epithelial cells.

ATR1 but not ATR2 mediate the effects of Ang II on Pgp function:

To examine whether the effects of Ang II are dependent on Ang II receptor 1 (ATR1), we examined the influence of Ang II on Pgp activity in the presence of the specific ATR1 antagonist Losartan K (100 μ M). The inhibitory effects of Ang II on Pgp function were abrogated in the presence of the antagonist (Figure 2). Interestingly, specific agonist of ATR2, CGP 42112 (2 μ M) did not mimic Ang II effects (**ATR2 agonist: 105 $\pm$ 15.28% Vs Control: 100%, p>0.05**). These data indicate that the effects of Ang II on Pgp function occur through ATR1.

Ang II effects on Pgp activity in Caco2 cells are PI3 kinase, Akt and p38 MAPK dependent:

Previous studies have shown that binding of Ang II to the Ang II receptor 1 (ATR1) is mediated primarily through Gq/11 signaling resulting in the activation of signaling proteins in IECs^{16,23–26}. Therefore, we examined the role of signaling mediators such as PI3 kinase (PI3K), Akt, p38 MAPK and ERK1/2 MAPK in the inhibition of Pgp function by Ang II. As shown in Figure 3, specific inhibitors of PI3K (LY294002; 25 μ M) or Akt (Triciribine; 1 μ M) or p38 MAPK (SB203580; 10 μ M) blocked the inhibitory effects of Ang II on Pgp activity in Caco2 cells. However, ERK1/2 MAPK inhibitor, U0126 (10 μ M) failed to block the effects of Ang II (Figure 3) suggesting the involvement of PI3K, Akt and p38 MAPK in Ang II-mediated inhibition of Pgp function. It should be noted that inhibitors alone did not show any change on Pgp function (data not shown).

Sequence of PI3K, Akt and p38 MAPK in Ang II-induced signaling pathway:

Previous studies in vascular smooth muscle cells (VSMCs) have shown that p38 MAPK is a downstream effector of PI3K in Ang II-induced signaling cascade²⁷. We next examined whether similar sequence of signaling event occurs in intestinal Caco2 cells. We first assessed the phosphorylation levels or activation of Akt and p38 MAPK by Ang II in the presence or absence of the PI3K inhibitor, LY294002. As shown in Figure 4A & B, Ang II significantly increased the phosphorylation of both Akt & p38 MAPK by ~2 fold as early as 15 min which persisted till 60 min compared to untreated control. However, as expected in the presence of LY294002, Ang II-induced phosphorylation of Akt was blocked (Figure 4A) suggesting that Akt is downstream to PI3K. Interestingly, LY294002 also abrogated the phosphorylation of p38 MAPK by Ang II (Figure 4B) indicating that p38 MAPK is also downstream to PI3K. Similar studies were performed with the p38 MAPK inhibitor, SB203580. As shown in (Figure 4C), the increase in Akt phosphorylation by Ang II at both 15 and 60 min time points was abrogated in the presence of SB203580 suggesting that Akt is downstream to p38 MAPK. As expected, SB203580 also blocked the increase in p38 MAPK phosphorylation by Ang II (data not shown). These results show that both p38 MAPK and Akt are downstream effectors of PI3K in the Ang-II induced signaling cascade.

Ang II decreases surface Pgp expression in Caco2 cells:

Mechanisms of rapid regulation for several membrane transporters such as Glut2²⁸, Glut4²⁹, NHE3³⁰ and DRA³¹ have been previously shown to involve membrane trafficking events. Involvement of PI3K-dependent pathway suggest that the rapid decrease in Pgp function by Ang II might be associated with changes in its levels on plasma membrane^{30,32–34}. We next examined the effects of Ang II (10 nM, 60 min) on surface levels of Pgp by cell surface biotinylation studies. Our results showed that Ang II treatment significantly decreased the surface levels of Pgp (170 kDa), whereas the total cellular Pgp levels did not change (Figure 5). These data are in parallel with a decrease in Pgp activity in Caco2 cells (Figure 1A). Densitometric analysis of the protein bands suggested that Ang II treatment decreased surface Pgp levels by 30–40% compared with control (Figure 5). These results suggest that Ang II under short-term conditions decreased surface Pgp levels via a membrane trafficking mechanism.

Akt suppression abrogates Ang II-induced decrease in surface Pgp expression:

Since PI3K, p38 MAPK & Akt were shown to be involved in mediating the effects of Ang II on Pgp function in Caco2 cells and that Akt was found to be downstream to PI3K and p38 MAPK in the Ang II-induced signaling cascade, we next examined whether Akt is involved in the decrease in surface Pgp levels in response to Ang II. For these studies, we performed cell surface biotinylation studies in untreated or Ang II treated Caco2 cells in the presence or absence of Akt inhibitor, triciribene. As shown in Figure 6, Ang II treatment significantly decreased the surface levels of Pgp (170 kDa) compared to untreated control, whereas the total cellular Pgp levels did not change. Moreover, the decrease in Pgp surface expression by Ang II was blocked in the presence of the Akt inhibitor (Figure 6), while the inhibitor alone did not show any effect (data not shown). Densitometric analysis of the protein bands suggested that Ang II treatment decreased surface Pgp levels by 30–40% compared with control. However, the decrease in Pgp surface expression was abrogated in the presence of the Akt inhibitor, triciribene (Figure 6). These results further suggest the role of Akt in Ang II induced decrease in Pgp levels on the plasma membrane.

Ang II decreases surface expression of Pgp via decreased exocytosis in Caco2 cells:

Decrease in the surface expression of Pgp could be due to a decrease in the exocytosis of Pgp or an increase in the endocytosis of Pgp. Therefore, first we examined whether the decrease in Pgp surface expression by Ang II resulted from decreased Pgp exocytosis. Exocytosis assays were performed in post-confluent Caco2 cells³⁵ in the presence and absence of Ang II. Cells were incubated with sulfo NH-SS-acetate to mask cell surface proteins followed by treatment with Ang II (10 nM) at 37°C for 60 min. The newly exocytosed proteins were assessed by cell-surface biotinylation. Figure 7 shows that Ang II decreased Pgp exocytosis as compared to untreated control cells. Densitometric analysis of the protein bands suggested that Ang II treatment decreased exocytosed Pgp levels by ~45% compared with control (Figure 7). These results further indicate that Ang II decreases Pgp surface expression perhaps via decreased exocytic insertion of Pgp.

Ang II decreases Pgp levels on the luminal membrane of ileum and colon of mice:

Since, Pgp is expressed in the ileum and colon of mice, in vivo biotinylation studies were performed to examine the short-term effects of Ang II on Pgp membrane levels in the ileum and colon. The levels of Pgp on the luminal membrane were significantly decreased in both the ileum (Figure 8A) and colon (Figure 8B) in response to Ang II. These results further suggest that Ang II-mediated inhibition of Pgp function in the native intestine is associated with a decrease in the membrane levels of Pgp. These results suggest that inhibition in the membrane levels of Pgp protein might be a major contributor to the observed pro-inflammatory effects of Ang II in the intestine.

DISCUSSION:

Ang II exerts a large number of physiological effects, including effects on vascular tone, hormone secretion, tissue growth and intestinal epithelial fluid and electrolyte transport^{15,20}. All components of the renin-angiotensin system (RAS) are present within the small intestine, and production of Ang II does occur locally within this tissue³⁶. For example, both

angiotensinogen (renin substrate) and renin (converts angiotensinogen to Ang I) have been shown to exist in enteric arteries and is synthesized within and released from the intestine. Further, ACE (angiotensin converting enzyme), which, plays an important role in conversion of Ang I to Ang II has been shown to be present in intestinal epithelial cells and in the endothelial lining of mesenteric vessels³⁷. Thus, Ang I formed within the intestine can be hydrolyzed rapidly to Ang II. The involvement of ACE and potentially renin with enterocytes may suggest a role for locally formed Ang II in the regulation of the various physiological processes in the intestine. In addition to the potential for the formation of Ang II in epithelial cells and enteric blood vessels observed in humans and rodents, Ang II-like immunoreactivity has also been found to be localized within enteric nerve terminals (myenteric and submucosal plexus)³⁶. Based on these observations it appears that Ang II is produced in vivo in epithelial cells, endothelial lining of blood vessels as well as submucosa. However, studies in the last few years have documented several non-hemodynamic effects of Ang II including pro-inflammatory and pro-fibrotic activities^{11,38}. In the present study, we demonstrated a novel role of Ang II in the inhibition of Pgp in intestinal epithelial cells (IECs) via a post-translational mechanism involving membrane trafficking events. These findings are of clinical importance, as elucidating mechanisms involved in the down-regulation of Pgp by Ang II underlie the pathophysiology of gut inflammatory disorders such as IBD in which Pgp function and expression is decreased.

Ang II is known to exhibit its effects by binding to two distinct G-protein coupled membrane receptors, namely Ang II receptor 1 and 2 (ATR1 and ATR2). Humans express a single ATR1, while two isoforms, ATR1_a and ATR1_b with 95% of amino acid sequence identity can be found in rat and mouse. ATR1_a, a homolog to the human ATR1, is expressed in the kidney, heart, brain, liver and intestine^{11,15}. ATR1_b is predominantly expressed in the anterior pituitary gland and adrenal zona glomerulosa^{11,15}. Ang II receptor subtype 2 (ATR2) is ubiquitously expressed in developing fetal tissues, decreases after birth and remains low in adult²¹. Both ATR1 and ATR2 have been shown to be present in the human small intestine, colon and intestinal epithelial Caco2 cells^{15,20}. However, studies regarding the basolateral vs apical expression of ATR1 in intestinal epithelial cells are not very conclusive. In this regard, earlier studies of Hirasawa et al³⁹ have shown that ATR1 is predominantly localized at the basolateral surface of the human colonic epithelium. However, in the rodent intestine, ATR1 was found to be expressed along the entire villus length at both brush border (BBM or apical) and basolateral membranes⁴⁰. Another study by Musch et al,²⁰ demonstrated the regulation of another intestinal transporter, NHE3 by Ang II in Caco2BBE cells (subclone of Caco2 cells). The authors showed that Caco2BBE cells treated with Ang II from the basolateral side significantly increased apical NHE3 activity. In addition, they also showed that when Ang II was added to the serosal side of segments of mouse jejunum mounted in Ussing chambers, the mucosal to serosal absorptive flux (indicative of apical NHE3 activity) was significantly increased. These studies support our observations that Caco2 cells treated basolaterally with Ang II showed a significant decrease in Pgp activity.

Recent studies have shown that ATR1 plays an important role in the pathogenesis of intestinal inflammation¹⁹. Furthermore, it is generally accepted that ATR2 exerts actions opposite to ATR1 and has been ascribed a 'tissue protective' role⁴¹. Our data demonstrated

that Ang II decreased Pgp function via the involvement of ATR1 but not ATR2, further suggesting that Ang II binds to ATR1 to exert its pro-inflammatory effects on Pgp function in intestinal Caco2 cells. Studies of Ewert et al,⁴² have shown that ATR2 has an ability to internalize and thus does not reach the surface membrane until under some instances such as inflammation. However, these observations do not hold true with the recent studies of Sun et al,⁴³, showing that treatment of Caco2 cells with Ang II for 48h, induced apoptosis through GATA-6 and the Bax pathway in an ATR2-dependent manner. Thus, these studies indicate that ATR2 is not internalized and is present on the surface membrane even after 48h of Ang II treatment. Our current study treating Caco2 cells with ATR2 agonist, CGP42112 for 1h further prove that effects of Ang II on Pgp activity are not mediated via ATR2 and that ATR2 is present on the surface membrane.

Previous studies have shown that binding of Ang II to ATR1 resulted in the activation of signaling proteins including PI3K/Akt, p38 MAPK or ERK1/2 MAPK signaling in IECs^{16,23,26}. However, our data showed that Ang-II induced inhibition of Pgp function was dependent on PI3K/Akt & p38 MAPK signaling pathway but did not involve ERK1/2 MAPK pathway. Moreover, our studies also demonstrated that both p38 MAPK along with Akt are downstream to PI3K in Ang II-induced signaling cascade in intestinal Caco2 cells. These results are consistent with studies in vascular smooth muscle cells (VSMCs), showing involvement of Akt and p38 MAPK pathway in response to Ang II²⁷. Furthermore, our data suggested that inhibition of Pgp function in IECs by the pro-inflammatory peptide, Ang II did not occur via an ERK1/2 MAPK signaling pathway. In this regard, our previous studies have demonstrated that the stimulation of Pgp function by the well-known anti-inflammatory agents, e.g. keratinocyte growth factor-2 (KGF2) and the probiotic *Lactobacillus acidophilus* was dependent on ERK1/2 MAPK^{44,45}. Thus, it seems that Erk1/2 MAPK signaling plays an important role in the up-regulation rather than inhibition of Pgp function in IECs.

Previous studies have shown that PI3 kinase is involved in regulating agonist-stimulated membrane trafficking events in mammalian cells^{30,32–34}. Furthermore, our studies also suggested that the involvement of PI3K in mediating the inhibitory effects of Ang II on Pgp function in Caco2 cells might be associated with changes in the levels of Pgp protein on the plasma membrane. Also, a number of studies have shown that membrane transport proteins are regulated via recycling events under short-term (acute) conditions between intracellular and apical membrane compartments (membrane trafficking)^{28–31}. Our results showed that Ang II-mediated inhibition of Pgp occurred via a significant decrease in the apical membrane levels of Pgp with almost no significant changes in the total levels of Pgp in Caco2 cells. These results suggest that Ang II inhibits Pgp in Caco2 cells via membrane trafficking events at the post-translational level. Further, the decrease in the surface expression of Pgp by Ang II was abrogated in Caco2 cells in the presence of the Akt inhibitor, triciribene suggesting that inhibition of Akt attenuated Ang II-mediated decrease in Pgp activity by abolishing Ang II-induced decrease in Pgp membrane levels. Also, our low stringent analysis of Pgp using in silico analysis by Scansite 2.0⁴⁶ identified potential phosphorylation sites for Akt. We speculate that these sequence motifs may play an important role in the direct or indirect phosphorylation of Pgp that could result in decreased Pgp function and membrane expression in response to Ang II. Further studies are needed to address this important issue.

Our data also showed that Ang II modulates Pgp recycling in IECs via decreased Pgp exocytosis. The role of Rab proteins in Pgp trafficking and recycling is unclear and is possibly dependent on the cell line used. Earlier studies have shown that Rab 11 regulates apical recycling of Pgp⁴⁷ and BSEP (Bile Salt Export Pump, ABCB11), or sPgp (sister protein of P-glycoprotein)⁴⁸ in polarized WIFB9 hepatoma cells. Furthermore, studies showed that Rab 11a was required for CFTR (another ABC transport protein, ABCC7) to undergo trafficking to the apical membrane in polarized intestinal epithelial cells⁴⁹. However, in non-polarized cells, studies have shown that Rab5 regulates Pgp exocytosis⁵⁰, as overexpression of dominant-negative mutant of Rab5 increased intracellular P-glycoprotein accumulation. Thus, it seems that, Rab 11 rather than Rab 5 may play an important role in apical recycling of Pgp in polarized intestinal epithelial cells. Whether Ang II/ATR1 modulates Pgp exocytosis via Rab 11 is not known and warrants further investigation.

We further validated our *in vitro* results of Caco2 cells using an *in vivo* mouse model as it reflects the complexity of the native intestine in a physiological setting. Consistent with the *in vitro* cell surface biotinylation results, our *in vivo* data showed that the levels of Pgp on the luminal membrane were significantly decreased in both the ileum and colon in response to short-term treatment of Ang II. It should be noted that for our *in vivo* studies, mice were administered with [Val⁵]-Ang II acetate salt hydrate instead of Ang II, as it is a more stable peptide compared to nascent Ang II peptide and is resistant to rapid degradation *in vivo*. Moreover, [Val⁵]-Ang II acetate is an ATR1 agonist and thus these *in vivo* studies further confirm the involvement of ATR1 in the observed inhibition of Pgp membrane expression in the ileum and colon.

In conclusion, our studies provide novel data on the inhibition of Pgp function and membrane expression by Ang II via a post-translational mechanism (Figure 9). Our findings showed that Ang II binds to ATR1 to activate a signaling cascade involving kinases such as PI3K, p38 MAPK and Akt. Both p38 MAPK and Akt were found to be downstream to PI3K in the Ang II-induced signaling pathway. Additionally, pharmacological inhibition of the downstream effector molecule, Akt abrogated the inhibitory effects of Ang II on Pgp function and associated reduction in Pgp protein levels on the plasma membrane. Ang II-induced reduction in Pgp surface expression in Caco2 cells was shown to occur via modulation of membrane trafficking events involving inhibition in the recycling of Pgp from the intracellular compartments to the apical membrane resulting in decreased Pgp exocytosis (Figure 9). These findings will provide the mechanistic basis for the pro-inflammatory effects of Ang II on the inhibition of Pgp membrane expression and function that might underlie pathophysiology of intestinal inflammation. Under inflammatory conditions, such as IBD, there is compromised intestinal epithelial barrier function due to dysregulated tight junction proteins. In addition, the endothelial junctions of the blood vessels at the site of inflammation are more dilated resulting in a leaky vasculature⁵¹ allowing access of macromolecules to the basolateral surface. Thus, we believe that targeting Angiotensin II type 1 receptors (ATR1) located at the basolateral surface by ATR1 blockers (ARBs)⁵² would be beneficial in treating IBD by attenuating the Angiotensin II mediated inhibition of Pgp. Thus, modulation of Pgp (MDR1) by Ang II/ATR1 appears to be a novel potential target for the treatment of gut inflammatory disorders including IBD.

MATERIALS AND METHODS:

Materials:

Caco2 cells were obtained from ATCC (American Type Culture Collection, Manassas, VA). ^3H -Digoxin (40 Ci/mmol) was purchased from Perkin-Elmer Life Sciences (Boston, MA). Substrate digoxin, Pgp/MDR1 inhibitor, verapamil, Angiotensin II and [Val⁵]-Angiotensin II acetate salt hydrate and ATR1 antagonist, Losartan K were obtained from Sigma (St. Louis, MO). PI3 kinase (LY294002), p38 MAP kinase (SB203580), Akt (Triciribene) and ERK1/2 MAP kinase inhibitors, PD98059 & U0126 were obtained from Biomol (Plymouth Meeting, PA). ATR2 agonist, CGP42112 was procured from Tocris Biosciences (Minneapolis, MN). Mouse monoclonal MDR1 antibody, goat anti-mouse and goat anti-rabbit antibody conjugated to horseradish peroxidase were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Phospho specific antibodies against p38 MAP kinase or Akt were obtained from Cell signaling (Boston, MA). Lipofectamine 2000, alexa fluor 488-conjugated goat antimouse secondary antibody and rhodamine phalloidin were procured from Invitrogen (Carlsbad, CA). All other chemicals were of at least reagent grade and were obtained from either Sigma Chemicals (St. Louis, MO) or Fisher Scientific (Pittsburgh, PA).

Cell culture and treatment:

Caco-2 cells were grown in T-75 (75 cm²) plastic flasks at 37°C in a 5% CO₂ environment. The culture medium consisted of EMEM (ATCC), 20% fetal bovine serum, 20 mM HEPES, 100 IU/ml penicillin, and 100 µg/ml streptomycin. Cells used for these studies were between passages 25 and 45 and were plated on 12-well Transwell inserts at a density of 1×10^4 cells/well and used for experiments at day 21st after plating as described previously^{44,45}. Fully differentiated Caco-2 monolayers were treated with Ang II at 10 nM concentration for 15, 30 or 60 min or with different concentrations of Ang II (0.1–10 nM) for 60 min from the basolateral side in the serum-free cell culture medium supplemented with 0.2% bovine serum albumin (BSA). In separate sets of experiments, cells were pre-treated with specific Ang II receptor 1 antagonist (ATR1), Losartan K (100 µM) or PI3K (LY294002; 25 µM), p38 MAPK (SB203580; 10 µM), Akt (Triciribine; 1 µM) or ERK1/2 MAPK (U0126; 10 µM) inhibitors for 1h and then co-incubated with Ang II (10 nM) for additional 1h or Caco2 cells were treated with the specific ATR2 agonist, CGP42112 for 1h.

Pgp-dependent digoxin flux activity:

Pgp activity was determined by measuring verapamil-sensitive ^3H -digoxin flux in Caco2 cells as previously described^{44,45}. Transport studies were performed in triplicate in the apical-to-basolateral (ab) and basolateral-to-apical (ba) directions with flux buffer containing ^3H -digoxin (1 µCi/ml supplemented with 1 µM unlabeled digoxin) from either the apical (200 µl) or basolateral (500 µl) compartments at room temperature in the presence or absence of Pgp inhibitor verapamil (10 µM). Samples (100 µl) were taken from receiver compartments (initial flux) at 15 min and then (final flux) at 60 min. The radioactivity of the receiver samples was determined using a Packard Tri-Carb 1600TR Liquid Scintillation analyzer (Packard Instruments: Perkin Elmer). The ab or ba values of the final flux were subtracted from the initial flux and digoxin flux activity was expressed as a ratio of verapamil-sensitive ba flux to ab flux.

Cell surface biotinylation:

Cell surface biotinylation studies were performed in untreated or Ang II-treated Caco-2 monolayers in the presence or absence of the Akt inhibitor, Triciribine; (1 μ M) utilizing Sulfo-NHS-SS-Biotin (1.5 mg/ml; Pierce) in borate buffer (in mM: 154 NaCl, 7.2 KCl, 1.8 CaCl₂, 10 H₃BO₃, pH 9.0) as described previously³¹. Labeling was allowed to proceed at 4°C to prevent endocytosis and internalization of antigens for 60 min. The biotinylated antigens were immunoprecipitated utilizing streptavidin agarose beads, and the biotinylated proteins were released by boiling in Laemmli buffer containing 100 μ M dithiothreitol. Proteins were subjected to 7% SDS polyacrylamide gel and then transferred onto nitrocellulose membrane. Nitrocellulose membranes were then utilized for immunoblotting with anti-MDR1 (Pgp) antibody^{44,45}. After 1h of incubation in blocking buffer (1X PBS and 5% nonfat dry milk), membranes were then incubated with the monoclonal MDR1 antibody (1:200 dilution) in the blocking buffer containing 1X PBS and 1% nonfat dry milk overnight at 4°C. Membranes were then incubated with horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG antibody (1:2000 dilution) for 1h at room temperature and washed for 30 min with agitation, during which the wash buffer (1X PBS and 0.1% Tween-20) was changed every 5 min. Bands were visualized with enhanced chemiluminescence (ECL) detection reagents. The surface Pgp levels were compared with total cell antigen as determined by immunoblotting in solubilized cell extract.

Exocytosis of Pgp:

To measure the exocytic insertion of Pgp into plasma membrane, NHS reactive sites on the Caco-2 cell surface were masked by pretreatment with sulfo-NHS-acetate at 4°C for 60 min, followed by quenching in 1 M glycine as previously described³⁵. Cells were then treated with Ang II (10 nM) at 37°C for 60 min and were subjected to cell-surface biotinylation as described above. The biotinylated fractions, which represent the newly inserted membrane proteins, were precipitated with neutravidin agarose beads and the precipitate was subjected to 7% SDS polyacrylamide gel and western blotting with anti-MDR1 (Pgp) antibody as described above.

Western blotting:

Caco-2 cells grown on transwell inserts were pre-treated with specific PI3K (LY294002; 25 μ M), p38 MAPK (SB203580; 10 μ M) or Akt (Triciribine; 1 μ M) inhibitors for 1h and then co-incubated with or without Ang II (10 nM) for additional 1h. Total cell lysates were then prepared as described previously^{44,45}. Briefly, after treatment, cells were washed with ice-cold 1X-PBS to remove residual media. Total protein was extracted by suspending the cell pellet in cell lysis buffer (Cell Signaling, Danvers, MA) supplemented with protease inhibitor cocktail (Roche, Indianapolis, IN). The cells were lysed by sonication (three pulses for 20 s each) and the lysate was centrifuged at 13000 rpm for 7 min at 4°C to remove cell debris. The supernatant containing the total cell proteins was collected and protein concentration was determined by Bradford method (29). Equal amounts (50 μ g/sample) of whole cell lysates were solubilized in SDS-gel loading buffer and boiled for 5 min. Lysates were run on a 10% or 12% SDS polyacrylamide gel and then transferred onto nitrocellulose membrane. Nitrocellulose membranes were then utilized for immunoblotting with phospho

Akt (Ser473) or phospho p38 MAPK (Thr180/Tyr182) or total Akt or p38 MAPK antibody. After 1h of incubation in blocking buffer (1X TBS and 3% BSA), the membranes were probed with phospho p38 MAPK or Akt antibodies (1:100 dilution) in 1X TBS, 1% BSA and 0.1% Tween-20 overnight at 4°C. The membrane was washed five times with the wash buffer (1X TBS and 0.1% Tween-20) for 5 min and probed with HRP-conjugated goat anti-rabbit antibody (1:2000 dilution) for 1 h followed by ECL (enhanced chemiluminescence, from Bio-Rad, Hercules, CA) detection. The blots were stripped to probe with total p38 MAPK or Akt antibodies.

***In vivo* studies:**

Animals: 8-week old male C57BL/6J mice were obtained from Jackson laboratories (Bar Harbor, Maine). Mice were given drinking water and standard rodent pellets ad libitum. Animal studies were conducted with prior approval of the Animal Care Committee of the University of Illinois at Chicago and Jesse Brown Veteran Affairs Medical Center. Mice were divided into two groups: 1) Vehicle group (n = 3) and 2) Ang II-treated group (n = 3). The two groups were administered vehicle or [Val⁵] Ang II acetate (2 mg/kg body wt, i.p.) for 2h, respectively, as described previously¹⁷. Mice were utilized for *in vivo* biotinylation studies.

***In vivo* biotinylation:**

To examine the luminal membrane localization of Pgp in mice administered Ang II, *in vivo* biotinylation was performed in the ileal and colonic regions of vehicle and Ang II treated mice utilizing native intestine of live animals (anesthetized) according to the procedure described for labeling luminal surface proteins in mouse jejunal loops⁵³. Biotinylated luminal surface proteins in mucosal lysates were then pulled down by streptavidin-agarose beads by incubating the lysates for 6h. Proteins were solubilized in Laemmli buffer, separated by 7% SDS polyacrylamide gel and probed with anti-Pgp antibody as described above

Statistical analysis:

Results are expressed as Mean $\pm$ SEM of three to five independent experiments. Students 't' test or one-way Anova with Tukey's test was used for statistical analysis. $P < 0.05$ or less was considered statistically significant.

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Figure 1. Short-term effects of Ang II on Pgp activity in Caco2 cells:

Overnight serum starved post-confluent Caco2 cells were treated with **A)** Ang II (10 nM) at different time points (15, 30 & 60 min) or **B)** different doses of Ang II (0.1–10 nM) for 60 min in serum free cell culture medium supplemented with 0.2% BSA for 60 min. [^3H]-digoxin flux (1 μM) was measured as described in Materials and Methods. Results are expressed as % of control and represent Mean $\pm$ SEM of 3 separate experiments. * $p < 0.05$; † $p < 0.01$ compared to untreated control.

Figure 2. Effects of Ang II on Pgp activity in Caco2 cells are Ang II receptor 1 (ATR1) dependent:

Overnight serum-starved post-confluent Caco2 cells were pre-treated with specific ATR1 antagonist, Losartan K (100 μ M) for 60 min in the serum free cell culture medium and then co-incubated with Ang II (10 nM) in serum free cell culture medium supplemented with 0.2% BSA for 60 min. [³H]-digoxin flux (1 μ M) was measured as described in Materials and Methods. Results are expressed as % of control and represent Mean $\pm$ SEM of 4 separate experiments. * p <0.05 compared to untreated control.

Figure 3. Effect of different kinase inhibitors on Ang II-induced inhibition of Pgp activity in Caco2 cells:

Overnight serum-starved post-confluent Caco2 cells were pre-treated with specific inhibitors of PI3K, LY294002 (25 μ M); p38 MAPK, SB203580 (10 μ M); Akt, Triciribine (1 μ M) and ERK1/2 MAPK, U0126 (10 μ M) for 60 min in the serum free cell culture medium and then co-incubated with Ang II (10 nM) in serum free cell culture medium supplemented with 0.2% BSA for 60 min. [³H]-digoxin flux (1 μ M) was measured as described in Materials and Methods. Results are expressed as % of control and represent Mean $\pm$ SEM of 4 separate experiments. * p <0.05 compared to untreated control.

Figure 4. p38 MAP kinase and Akt are downstream to PI3 kinase in Ang II-induced signaling cascade:

Overnight serum-starved post-confluent Caco2 cells were treated with Ang II (10 nM) for 5, 15 & 60 min in the serum free cell culture medium or pre-treated with specific inhibitors of PI3K, LY294002 (25 μ M) (A & B) & p38 MAPK, SB203580 (10 μ M) (C) for 60 min in the serum free cell culture medium and then co-incubated with Ang II (10 nM) in serum free cell culture medium supplemented with 0.2% BSA for 5, 15 & 60 min. After washing the cells with 1X PBS, extracted proteins (75 μ g) were subjected to Western blot analysis on 10 or 12% SDS-polyacrylamide gel utilizing phospho-specific Akt antibody (pAkt) (A & C) or p38 MAPK (pp38 MAPK) (B). The blots were stripped and re-probed with the Akt (total Akt) or p38 MAPK antibody (total p38 MAPK) to indicate equal loading of protein in each lane. A representative blot of 3 different experiments is shown. The data were quantified by densitometric analysis and expressed as arbitrary units and represent Mean $\pm$ S.E.M of 3 separate experiments. * $p < 0.05$; † $p < 0.01$ compared to untreated control (0 min).

Figure 5. Ang II decreases Pgp surface expression:

Overnight serum starved post-confluent Caco2 cells were treated with Ang II (10 nM) in serum free cell culture medium supplemented with 0.2% BSA for 60 min and subjected to cell surface biotinylation at 4°C using sulfo-NHS-SS-biotin. After solubilization, biotinylated proteins were extracted with streptavidin-agarose from equal amounts of total cellular protein. Surface and total cellular fractions were run on 7% SDS-polyacrylamide gel electrophoresis followed by transfer to nitrocellulose membrane. The blot was immunostained with a mouse Pgp/MDR1 antibody (Pgp). A representative blot of 3 different experiments is shown. The data were quantified by densitometric analysis and expressed as arbitrary units and represent Mean $\pm$ S.E.M of 3 separate experiments. * $p < 0.05$ compared to untreated control.

Figure 6. Ang II-induced decrease in Pgp surface expression is dependent on Akt activation: Overnight serum-starved post-confluent Caco2 cells were pre-treated with specific Akt inhibitor, triciribine (1 μ M) for 60 min in the serum free cell culture medium and then co-incubated with Ang II (10 nM) in serum free cell culture medium supplemented with 0.2% BSA for 60 min and subjected to cell surface biotinylation at 4°C using sulfo-NHS-SS-biotin. After solubilization, biotinylated proteins were extracted with streptavidin-agarose from equal amounts of total cellular protein. Surface and total cellular fractions were run on 7% SDS-polyacrylamide gel electrophoresis followed by transfer to nitrocellulose membrane. The blot was immunostained with a mouse Pgp/MDR1 antibody (Pgp). A representative blot of 3 different experiments is shown. The data were quantified by densitometric analysis and expressed as arbitrary units (surface Pgp/total Pgp) and represent Mean $\pm$ S.E.M of 3 separate experiments. * p <0.05 compared to untreated control.

Figure 7. Ang II-induced decrease in Pgp surface expression occurs via decreased exocytosis of Pgp:

Overnight serum-starved post-confluent cell-surface Caco-2 cells were masked with NH-SS-acetate at 4°C for 60 min, followed by quenching in 1 M glycine. Cells were then treated with Ang II (10 nM) in serum free cell culture medium supplemented with 0.2% BSA at 37°C for 60 min. The amount of newly exocytosed protein was assessed by cell-surface biotinylation. Biotinylated and total cellular fractions were run on 7% SDS-polyacrylamide gel electrophoresis followed by transfer to nitrocellulose membrane. The blot was immunostained with a mouse Pgp/MDR1 antibody (Pgp). A representative blot of 3 different experiments is shown. The data were quantified by densitometric analysis and expressed as arbitrary units (exocytosed Pgp/total Pgp) and represent Mean $\pm$ S.E.M of 4 separate experiments. [†]p<0.01 compared to untreated control.

Figure 8. Ang II decreases Pgp membrane expression in ileum and colon of mice:

Mice were administered Ang II (2 mg/kg body wt.) or vehicle (sterile water) intra-peritoneally for 2h and luminal membrane localization of Pgp was assessed by *in vivo* biotinylation studies as described in Materials and Methods. The biotinylated luminal surface proteins in the ileal (A) and colonic (B) mucosal lysates were pulled down by streptavidin-agarose beads by incubating the lysates for 6h. Proteins were then solubilized in laemmli buffer, separated by 7% SDS-PAGE and probed with Pgp antibody (Pgp). A representative blot is shown. Lower panel shows densitometric analysis of relative band intensities. Results represent Mean $\pm$ S.E.M of 3 mice. * $p < 0.05$ compared to vehicle (control).

Figure 9. Schematic of the proposed model of Ang II mediated effects on P-glycoprotein.