Angiotensin II Enhances Interleukin-18 Mediated Inflammatory Gene Expression in Vascular Smooth Muscle Cells

A Novel Cross-Talk in the Pathogenesis of Atherosclerosis

Saurabh Sahar, Roopashree S. Dwarakanath, Marpadga A. Reddy, Linda Lanting, Ivan Todorov, Rama Natarajan

Abstract—Vascular smooth muscle cells (VSMCs) express functional interleukin-18 receptors (IL-18Rs), composed of α and β subunits. These subunits are elevated in VSMCs of atherosclerotic plaques and can be induced by inflammatory agents in cultured VSMC. Because both IL-18 and Angiotensin II (Ang II) are implicated in atherosclerosis, our objective was to analyze the role of IL-18 signaling and potential cross-talk with Ang II in VSMC. We observed that IL-18 activated Src kinase, protein kinase C, p38 and JNK MAPKs, Akt kinase, transcription factors NF-κB and AP-1, and induced expression of pro-inflammatory cytokines in VSMC. Pretreatment of VSMC with Ang II enhanced IL-18-induced NF-κB activation and cytokine gene expression. Interestingly, Ang II directly increased mRNA and cell surface protein levels of the IL-18Rα subunit. Functional relevance in an organ culture model was demonstrated by the observation that incubation of intact mouse aortas ex vivo with Ang II also significantly increased IL-18Rα expression. Furthermore, Ang II significantly stimulated transcription from a minimal IL-18Rα promoter containing putative binding sites for STAT and AP-1. Ang II also increased in vivo recruitment of STAT-3 on the IL-18Rα promoter. Finally, dominant negative STAT-3 mutant blocked Ang II-induced IL-18Rα promoter activation in CHO cells overexpressing AT1a receptor and IL-18Rα mRNA expression in HSVSMC. Thus, Ang II enhances IL-18 induced inflammatory genes by increasing IL-18Rα expression. These results illustrate a novel mechanism wherein Ang II-mediated increases in inflammatory genes and proatherogenic effects in the vasculature are enhanced by a vicious loop and cross-talk with the IL-18 signaling pathway. (Circ Res. 2005;96:1064-1071.)

Key Words: angiotensin II ■ cytokines ■ interleukin-18 ■ NF-kB ■ vascular smooth muscle cells

Angiotensin II (Ang II) plays a crucial role in the atherogenic process not only through its pressor responses but also because of its growth promoting and inflammatory effects.1–4 Increased plasma concentrations of Ang II have been implicated in atherosclerosis. Ang II infusion greatly accelerated the development of atherosclerotic lesions and aneurysms in apolipoprotein E-deficient (apo E−/−) mice.5 Evidence suggests that interleukin-18 (IL-18) is also a proatherogenic cytokine. IL-18 is expressed in human carotid atherosclerotic plaques and this was related to plaque instability.6 Administration of exogenous IL-18 enhanced the development of atherosclerosis in apo E−/− mice through an interferon-γ (IFN-γ) dependent mechanism.7 Atherosclerotic lesion development was reduced in apo E−/− mice either by overexpressing IL-18 binding protein, the endogenous inhibitor of IL-188, or by IL-18 deficiency.9 It was recently reported that human atheroma expresses elevated levels of both IL-18 and IL-18 receptors compared with nondiseased arterial tissue.10 In atherosclerotic plaques macrophages express IL-18 whereas vascular smooth muscle cell (VSMC) and endothelial cells express both the α and β subunits of IL-18 receptor. In vitro, VSMC express modest basal levels of IL-18 receptor subunits, and their expression was enhanced by a combination of tumor necrosis factor-α (TNF-α), IL-1β, and IFN-γ treatment. On the other hand, a combination of IL-12 and IL-18 treatment increased the expression of IFN-γ in VSMC.10

IL-18 was first described as an IFN-γ-inducing factor.11 It is a key inducer of T helper 1 (Th1) cell development and function, activating both Th1 cells and natural killer (NK) cells. IL-18 receptors belong to the IL-1 receptor/Toll-like receptor superfamily.12 IL-18 signaling has been studied in depth in immune cells where it activates NF-kB and p38 mitogen activated protein kinase (p38 MAPK). This signaling involves IL-1 receptor associated kinases (IRAKs 1 and 4), and adaptor proteins like MyD88 and TRAF-6.13–18 However, the effects of IL-18 in VSMCs are still poorly understood. Furthermore, the relationships between the actions of IL-18...
and well-established VSMC agonists, such as Ang II, have not been studied.

In the present study we showed that IL-18 activates several key signaling pathways including MAPKs, transcription factors NF-kB and AP-1, and induces the expression of proinflammatory cytokines and chemokines such as Interleukin-6 (IL-6), IL-8 and Monocyte Chemoattractant Protein-1 (MCP-1). We also observed for the first time that pretreatment of VSMC with Ang II could enhance the effects of IL-18 on inflammatory gene expression by upregulating IL-18Rα subunit expression, and that STAT-3 plays a major role in this induction. Taken together, our new data demonstrate that IL-18 is a proinflammatory cytokine in VSMCs, and that Ang II and IL-18 mediated expression of inflammatory genes and development of atherosclerosis may be exacerbated by interactions between their signaling pathways.

Materials and Methods

Ex Vivo Organ Cultures of Intact Mouse Aortas

All animal studies were performed according to a protocol approved by our Research Animal Care Committee. Segments of thoracic aorta, 1 to 2 cm in length, were excised in a sterile manner from 11-week-old normal chow-fed C57BL/6 mice (Jackson Laboratories) and placed in cold medium. The artery specimens were carefully stripped clean of surrounding fat, rinsed extensively with phosphate-buffered saline (PBS), and then one half of each aorta was cultured in DMEM serum-depletion medium alone and the other half in DMEM serum-depletion medium containing 10⁻² mol/L Ang II for 4 hours. At the end of the incubation period, tissues were snap-frozen in liquid nitrogen and stored at −70°C. Tissues were disrupted using a mortar and pestle and homogenized using QIA shredder homogenizers, followed by RNA extraction as described later.

Cloning of the Human IL-18Rα Promoter

In order to clone the minimal promoter for human IL-18Rα, we scanned the Homo sapiens chromosome 2 genomic contig to examine the 1Kb region of genomic DNA immediately upstream of the IL-18Rα translation start site (GenBank accession NT_022171; sequence 5044134 to 5045134). A genomic DNA fragment containing human IL-18Rα promoter and 5’-upstream sequences (424 bp upstream of translation start site) was amplified using sense (5’-ACACACTCTGTATACATGGA-3’) and antisense (5’-GGTTTGGATTCTGCTTCAAAT-3’) primers and the product was directly cloned into a pCR3.1 vector using TA cloning kit. The insert sequence was verified by DNA sequencing. The IL-18Rα promoter fragment was released by digestion with KpnI and Xhol, and subcloned upstream of luciferase gene in pGL3 basic vector (Promega) that was also linearized by KpnI-XhoI digestion.

Chromatin Immunoprecipitation (ChIP) Assay

Human VSMC were either left untreated or treated with Ang II (10⁻⁷ mol/L) for indicated time periods. Cells were fixed with 1% formaldehyde. ChIP assays were then performed using a kit from Upstate, Inc. according to manufacturer’s protocols using 5 μL of anti-STAT3 antibody. An aliquot of the cell lysates was used to isolate total Input DNA. PCR amplification of the immunoprecipitated DNA was performed using the same set of primers used for cloning the 424 bp region of human IL-18Rα promoter.

Data Analyses

Results are expressed as means±SE of multiple experiments. Student t tests were used to compare 2 groups, or ANOVA with the Newman-Keuls post tests for multiple groups using Prism software (Graph Pad). Statistical significance was detected at the 0.05 level. Detailed Materials and Methods section is available in the online data supplement at http://circres.ahajournals.org.

Results

Activation of VSMC by IL-18

In order to evaluate the signal transduction mechanisms of IL-18 actions in VSMC, we examined whether it could activate key signaling pathways including protein kinase C (PKC), Src tyrosine kinase and MAPK family members that are involved in extracellular stress, growth, and inflammation. Serum-depleted VSMCs were treated with IL-18 (25 ng/mL) for indicated time periods and cell lysates assayed for MAPK activation by immunoblotting with phospho-specific antibodies. Figure 1A shows that IL-18 increased p38 MAPK activation by 30 minutes with peak at 1 hour. JNK was also similarly activated. However, ERK1/2 was not activated. The delayed activation of MAPKs suggests a possible secondary effect. We also found that IL-18 could activate Akt kinase (Figure 1A) in 30 minutes and this continued to increase up to 2 hours, suggesting that IL-18 may play a role in VSMC survival. On the other hand, IL-18 could activate Src kinase and PKC α/β by 5 minutes (Figure 1B). Activation of Src and PKC may lead to the activation of downstream MAPKs that play an important role in growth and inflammatory gene expression. Although IL-18 effects on MAPK activation were visible even at lower doses of IL-18 (10 ng/mL), the maximal effects were seen at 25 ng/mL to 50 ng/mL IL-18 (data not shown).

Activation of Transcription Factors and Inflammatory Gene Expression by IL-18

Because MAPKs, Akt, Src, and PKC can lead to the activation of key transcription factors such as AP-1 and NF-kB that are associated with inflammatory gene expression, we next evaluated whether IL-18 can activate AP-1 and NF-kB. We
examined the nuclear translocation of p65, the transcriptionally active subunit of NF-kB, by immunofluorescence using an anti-p65 antibody. Figure 2A shows that under basal conditions, p65 remained cytosolic, but 15 minutes after stimulation with IL-18 (25 ng/mL), p65 translocated into the nucleus, thus indicating that IL-18 can activate NF-kB in VSMCs.

To examine AP-1 activation, VSMCs were transiently transfected with a reporter plasmid AP-1-Luc containing 3x AP-1 binding sites upstream of the luciferase reporter gene, and luciferase activity determined after stimulation with IL-18. As shown in Figure 2B, AP-1 transcriptional activity was significantly increased by IL-18 (2-fold versus control, \( P < 0.05 \)).

To examine whether IL-18 induces proinflammatory genes controlled by these transcription factors, serum-depleted HVSMCs were stimulated with IL-18 (25 ng/mL) for 1 to 12 hours and inflammatory cytokine and chemokine gene expression determined by relative RT-PCR. Figure 2C shows that IL-18 can induce MCP-1 mRNA expression in 1 hour, and IL-8 and IL-6 mRNAs in 6 to 12 hours. Overall these results demonstrate that IL-18 is a proinflammatory cytokine in VSMC, and may enhance the atherogenic process by inducing the expression of other proinflammatory molecules.

Enhancement of IL-18 Induced Proinflammatory Gene Expression in Ang II Pretreated VSMCs

Because both Ang II and IL-18 have proatherogenic effects, we hypothesized that there could be a cross-talk and synergism between their signaling pathways. HVSMCs were pretreated with Ang II (10^{-7} mol/L) for 3 hours, washed to remove Ang II, and then stimulated with IL-18 for various time intervals. Activation of NF-kB and induction of IL-6 and IL-8 expression were then evaluated as markers of downstream functional end points of IL-18 signaling. We noted that NF-kB activation by IL-18 was clearly enhanced under these conditions (Figure 3A). Thus, IL-18 stimulated p65 nuclear translocation was seen as early as 5 minutes in VSMC that had been pretreated with Ang II compared with 15 minutes in cells without Ang II pretreatment (compare Figure 3A with Figure 2A).

We next examined the effect of Ang II pretreatment on IL-18 induced expression of proinflammatory cytokines and
chemokines. Results showed that in HVSMCs pretreated for 3 hours with Ang II, IL-6 mRNA expression was induced earlier and also enhanced compared with those without Ang II pretreatment (Fig. 3B, upper panel). This Ang II pretreatment also clearly enhanced IL-8-induced IL-8 mRNA expression (Figure 3B, lower panel). However, we did not observe any significant enhancement in MCP-1 expression (data not shown). IL-8 induced IL-6 protein levels (by ELISA) at the 6 hour time point were also significantly higher in the culture supernatants from cells pretreated with Ang II (Figure 3C). These data further confirmed that Ang II can significantly enhance the induction of proinflammatory genes by IL-18.

In order to evaluate any potential growth promoting effects, we next examined the effect of IL-18 and Ang II pretreatment on 3H-Leucine or 3H-Thymidine incorporation in PVSVC to determine the rates of protein and DNA syntheses, respectively. As shown in Figure 3D, IL-18 by itself could enhance 3H-Leucine incorporation (a measure of hypertrophy) by \( \approx 40\% \) \( (P < 0.01) \) and this was only slightly less than the direct effects of Ang II \( (\approx 70\%, P < 0.001) \). Pretreatment of cells with Ang II for 3 hours, followed by washing with PBS, still yielded a 54% increase in 3H-Leucine incorporation. However, there was no further enhancement of 3H-Leucine incorporation by IL-18 after the 3 hour pretreatment with Ang II. Unlike leucine incorporation, IL-18 had no effect on 3H-Thymidine incorporation (data not shown). These results indicate that Ang II-mediated potentiation of IL-18 effects is restricted to inflammatory gene expression, and does not include growth-promoting effects.

### Induction of IL-18Rα Expression by Ang II

In order to evaluate the mechanisms by which Ang II enhances IL-18 signaling, we hypothesized that Ang II can induce the expression of IL-18Rα. Quiescent HVSMCs were treated with Ang II \( (10^{-7} \text{ mol/L}) \) for 1 to 12 hours, RNA extracted and RT-PCR performed using primers specific for human IL-18Rα. Figure 4A shows that Ang II directly increased IL-18Rα mRNA expression by 1 hour, peaking at 3 hours after stimulation, and this was comparable to the positive control TNF-α. Ang II induced IL-18Rα mRNA expression was evident even at lower doses of Ang II \( (10^{-8} \text{ mol/L}) \), whereas maximal effects were observed at \( 10^{-7} \text{ mol/L} \) (data not shown). Figure 4B shows that Ang II at 3 hours significantly increased IL-18Rα mRNA expression \( (P < 0.01) \). Treatment with actinomycin D \( (10 \mu\text{mol/L}) \) blocked IL-18Rα mRNA induction by Ang II, suggesting that this effect is transcriptional (Figure 4C) \( (35\% \text{ inhibition}, n = 2) \). Furthermore, immunofluorescent staining of HVSMC with antihuman IL-18Rα antibody showed that Ang II also increased the cell surface-expression of IL-18Rα protein (Figure 4D). Intense staining for IL-18Rα was visible on the surface and the edges of Ang II treated HVSMC. These results demonstrate for the first time that Ang II induces the expression of both IL-18Rα mRNA and protein in VSMC.

In order to determine whether these in vitro findings are also observed ex vivo, we evaluated the effects of incubating intact mouse aortas with Ang II in an organ culture model. Thoracic aortas were excised from four 11-week-old normal chow-fed C57BL/6 mice. Each aorta was cut into smaller rings and one half of each aorta cultured in serum-depletion media with Ang II \( (10^{-7} \text{ mol/L}) \) for 4 hours, and the other half without Ang II (control). RNAs extracted from these blood vessels were then subjected to relative RT-PCR analyses using primers specific for mouse IL-18Rα and 18S as internal control. As shown in Figure 4E and 4F, Ang II could also increase the expression of IL-18Rα mRNA in mouse aortic tissues by over 2-fold \( (P < 0.03 \text{ by paired t-test}, n = 4) \).

### Activation of Human IL-18Rα Promoter by Ang II

To determine the nature of the cis-elements and trans-acting factors involved in Ang II mediated upregulation of IL-18Rα expression in VSMC, we performed DNA sequence analysis using TRANSFAC software which revealed the presence of 3 consensus STAT binding sites and 2 consensus AP-1 binding sites in the human IL-18Rα promoter (Figure 5A). We cloned for the first time a genomic DNA fragment containing hIL-18Rα promoter and 424 bp upstream of translation initiation site into a luciferase reporter plasmid (described under Methods). When the resultant reporter plasmid (pIL18Rα-424) was transiently transfected into

![Figure 4](http://circres.ahajournals.org/)
Involvement of the AT₁a Receptor and STAT-3 in Transcriptional Activation of IL-18Rα Promoter by Ang II

To further characterize the IL-18Rα expression and determine the role of AT₁a receptor we used a CHO cell line stably overexpressing AT₁a receptor (CHO-AT₁a) (a gift from Dr Eric Clauer). CHO-AT₁a cells, and control CHO cells without AT₁a (CHO-WT), were transiently transfected with pIL18Rα-424 and luciferase activities determined. Results showed that the basal level of transcription from pIL18Rα-424 was ~4-fold higher in CHO-AT₁a cells compared with CHO-WT cells (Figure 6A). This increase in basal activity of AT1 after overexpression is similar to that observed by Zou et al. who showed that overexpression of AT1 receptors in COS-7 cells increases basal inositol phosphate levels by 5 fold. In CHO-AT₁a cells Ang II (10⁻⁷ mol/L) further increased the promoter activity by 57%. In contrast, Ang II failed to stimulate pIL18Rα promoter in CHO-WT cells (Figure 6A). These results indicate that Ang II induced IL-18Rα promoter activation is via AT₁a. Figure 6B shows that Ang II activates the IL-18Rα promoter in CHO-AT₁a cells in a dose-dependent manner with maximum activation at 10⁻⁷ mol/L Ang II.

In order to confirm the involvement of STAT-3 in Ang II mediated transcription of IL-18Rα, CHO-AT₁a cells were cotransfected with pIL18Rα-424 and a STAT-3 dominant negative mutant vector. As shown in Figure 6C, this dominant negative STAT-3 significantly blocked Ang II mediated transcription from the minimal IL-18Rα promoter. Furthermore, nucleofection of HVSMC with dominant negative STAT-3 blocked Ang II induced IL-18Rα mRNA expression (Figure 6D). These results demonstrate that Ang II can induce the transcriptional activation of IL-18Rα via the AT₁a receptor and STAT-3 activation.

Discussion

Cytokines are important modulators of inflammatory events during all stages of atherogenesis. Although VSMCs express receptors for IL-18, their signaling mechanisms, functional consequences and potential inflammatory effects have not been well investigated. In this report we have demonstrated that IL-18 could activate Src, a nonreceptor tyrosine kinase that can regulate several downstream kinases as well as transcription factors and has been implicated in VSMC growth and migration. We also found that IL-18 could activate PKC and the MAPKs p38 and JNK that play key roles in the activation of transcription factors and regulation of inflammatory genes. PKC can also activate MAPK pathway, presumably by the phosphorylation of raf-1. However, the role of Src and PKC in the IL-18 stimulated activation of MAPKs needs further investigation.

Although IL-18 could activate Src and PKC in 5 to 15 minutes, MAPK activation was delayed (around 1 hour). ERK1/2 MAPK was not activated by IL-18, which corroborates with our unpublished observations that IL-18 had no direct mitogenic effects in VSMC. The specific reason for the delayed activation of MAPKs by IL-18 is not clear from the present studies. It is possible that MAPK activation is a secondary effect of IL-18 arising from the actions of some

PVS PMC, luciferase gene expression under the control of IL-18Rα promoter was significantly stimulated by 6 hour Ang II treatment (1.6 fold versus control, P<0.05) (Figure 5B). We next examined whether Ang II could activate the transcription factors whose binding sites were found on the hIL-18Rα promoter. Ang II activated transcription from an AP-1 luciferase reporter plasmid (Figure 5C) in transiently transfected VSMC. Using immunoblotting of cell lysates, we also confirmed that Ang II indeed phosphorylates both STAT-1 (at Tyr 701) and STAT-3 (at Tyr 705) (Figure 5D). These results suggest that STAT-1, -3, and AP-1 transcription factors may be involved in Ang II mediated induction of IL-18Rα. Finally, to verify whether Ang II can recruit transcription factors to the IL-18Rα promoter in vivo in the context of chromatin, we performed ChIP using a STAT-3 antibody. Figure 5E shows that after 3 hours of Ang II treatment, there is a marked increase in STAT3 occupancy on the IL-18Rα promoter.

Figure 5. Characterization of hIL-18Rα promoter. (A) Schematic diagram showing the putative transcription factor binding sites on hIL-18 Rα promoter. (B) PVS PMC were transiently transfected with pIL18Rα-424 plasmid and luciferase activity measured after stimulation with Ang II (10⁻⁷ mol/L) for 6 hour. Data represents mean±SE of 4 experiments performed in triplicate. (*, P<0.05 vs control) (C) PVS PMC were transfected with AP-1- Luciferase construct and luciferase activity determined after Ang II (10⁻⁷ mol/L) treatment for 6 hour. Data represents mean±SE of 3 experiments (*, P<0.005 vs control.) (D) Serum-depleted HVSMCs were either left untreated (Control) or stimulated with Ang II (10⁻⁷ mol/L) for 10 and 30 minutes, and p-STAT1 & p-STAT3 levels detected by immunoblotting. β-actin was used as a control for protein loading. Results shown are representative of 3 independent experiments. (E) Representative data of ChIP assay (n=2). Serum-depleted HVSMCs were either left untreated (Control) or stimulated with Ang II (10⁻⁷ mol/L) for 2 and 3 hours. Cells were fixed with formaldehyde and ChIPs performed with anti-STAT3 antibody. PCR was performed on immunoprecipitated DNA and Input DNA using IL-18Rα promoter primers.
factors made by IL-18. Although IL-18 did not increase DNA synthesis in VSMC, it significantly increased tritiated leucine incorporation, suggesting that it has hypertrophic effects similar to Ang II.

Activation of these signaling kinases may also be related to VSMC migration since, in unpublished studies, we observed that, although IL-18 did not directly induce VSMC migration in an in vitro Boyden chamber assay, it clearly augmented the chemotactic effects of platelet-derived growth factor-BB.

Activation of JNK and p38 MAPK by IL-18 may lead to transcriptional activation of early response genes such as c-fos and c-jun, which can further lead to gene expression via AP-1, NF-kB and other transcription factors. NF-kB is involved in inflammatory responses elicited by IL-18 in many cell types.17,18 NF-kB activation is a point of convergence by which several proinflammatory agents cause inflammation in the vessel wall. Several inflammatory genes are regulated by both NF-kB and AP-1. We have shown for the first time that IL-18 could activate both NF-kB and AP-1 in VSMC. Furthermore, proinflammatory cytokines IL-6, IL-8 and chemokine MCP-1, regulated by NF-kB and AP-1, were also induced by IL-18. These results demonstrate that IL-18 can induce the expression of proinflammatory genes in the vessel wall that may lead to the development of atherosclerosis.

Recent research has indicated prominent roles for Ang II and IL-18 in the pathogenesis of atherosclerosis.1–9 We have sought to delineate the interrelationship between Ang II and IL-18 in mediating inflammatory events in VSMCs because this could provide clues regarding the function of IL-18 in VSMC and also provide novel data on how proinflammatory cytokines and Ang II can cooperate to augment pathogenic effects in VSMCs. In order to investigate a potential crosstalk between Ang II and IL-18 signaling pathways, we evaluated the effects of pretreatment of VSMCs with Ang II on IL-18 signaling. Interestingly, we found that IL-18 effects on NF-kB activation and IL-6 and IL-8 induction were markedly enhanced after a 3 hour pretreatment with Ang II. However, MCP-1 expression was not further enhanced by Ang II pretreatment, indicating that this is restricted to a set of key VSMC inflammatory genes.

The IL-18 receptor complex consists of 2 receptor chains: ligand-binding IL-18Rα chain and signal-transducing IL-18Rβ chain.25 Importance of IL-18Rα was shown by transient transfection into COS-1 cells that conferred IL-18 binding properties to the cells and capacity for NF-kB activation.26 Moreover, Th1 cells and NK cells derived from IL-18Rα deficient mice showed impaired IL-18 signaling.27 A recent report supports the notion that IL-18 is a weak activator in most cell types and needs a costimulant that increases receptor expression.28 These arguments led us to hypothesize that enhancement of IL-18 signaling by Ang II pretreatment might be because of the induction of IL-18Rα expression on VSMC by Ang II. We showed for the first time that pretreatment of VSMC with Ang II clearly increased the expression of both IL-18Rα mRNA and cell surface protein expression. These in vitro results were further strengthened by our data with organ cultures demonstrating that Ang II can induce IL-18Rα mRNA expression ex vivo in mouse aortas.

In order to determine the trans-acting factors mediating Ang II-induced activation of the IL-18Rα promoter, we cloned for the first time a minimal region of the hIL-18Rα promoter, which contained STAT and AP-1 sites. We found that Ang II could significantly increase transcription from this minimal promoter in both PVSMC as well as in CHO-AT1a cells.

Ang II is known to activate the JAK-STAT pathway in several cell types.29 STAT-1 and STAT-3 in rat VSMCs30,31 and STAT-5 in rat cardiac myocytes and mesangial cells.32,33 In this report we confirmed that Ang II can activate STAT-1 and STAT-3 in HVSMC, whereas STAT-5 was not activated...
(unpublished observation). We also confirmed that Ang II can activate AP-1 as previously shown.\textsuperscript{34} ChIP assays confirmed that Ang II can increase the recruitment of STAT-3 to the IL-18\(\alpha\) promoter. Overexpression of dominant negative STAT-3 completely blocked Ang II induced transcription from the IL-18\(\alpha\)-Luc construct in CHO-AT\(_1\)cels, and also IL-18\(\alpha\) mRNA expression in HVSMC. Thus these results confirm that STAT-3 plays a crucial role in Ang II induced IL-18\(\alpha\) expression.

In vivo monocytes/macrophages trapped in the subendothelial space under atherogenic conditions may secrete IL-18 and other cytokines, which activate VSMC inflammatory gene expression, migration, and augment the actions of Ang II. Reciprocally, Ang II can augment the effects of these cytokines by directly increasing the expression of inflammatory genes induced by Ang II and proinflammatory cytokines such as IL-18, which activate VSMC inflammatory gene expression.

**Figure 7.** Schematic representation of the interaction of Ang II and IL-18 signaling pathways leading to inflammatory gene expression.

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<tr>
<th>Angiotensin II</th>
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<td>AT(_1) receptor</td>
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**References**


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**Materials.** Ang II was obtained from Peninsula laboratories (San Carlos, CA). Phospho-specific antibodies for STAT-1, STAT-3, p38, ERK, JNK/SAPK, AKT, Src, PKC α/β and antibody for total STAT-3 were from Cell Signaling (Beverly, MA). Anti-NF-kB p65 antibody was from Rockland Inc. (Gilbertsville, PA). IL-6 ELISA kit, recombinant human IL-18 and anti-human IL-18 Rα antibody were from R&D Systems (Minneapolis, MN). Restriction and modification enzymes were from New England Biolabs (Beverly, MA). Transfection reagent Lipofectamine 2000 and TA cloning kit were from Invitrogen (Carlsbad, CA). Nucleofector kit was from Amaxa Biosystems (Cologne, Germany). RNeasy columns, QIAshredder columns, and transfection reagent Effectene were from Qiagen (Valencia, CA). Reagents for immunofluorescence were from Molecular Probes (Eugene, OR). Quantum RNA 18S primers were from Ambion, Inc (Austin, TX) and cDNA kits from Applied Biosystems (Foster City, CA). Chromatin immunoprecipitation (ChIP) assay kit was from Upstate, Inc (Charlottesville, VA). Plasmid expressing dominant negative STAT-3 was a generous gift from Dr. Allan R. Brasier (University of Texas Medical Branch, Galveston) (1). The AP-1-Luc plasmid carrying three AP-1 binding sites upstream of the luciferase reporter gene was from Dr. Jian-Jian Li (Beckman Research Institute of City of Hope, Duarte). Promoterless pGL3b vector and luciferase assay reagents were from Promega (Madison, WI). CHO-AT1a cells (2,3) were a generous gift from Dr. Eric Clauser (INSERM EPI 0103, France).

**Cell Culture and treatment with Ang II or IL-18.** Primary cultures of porcine VSMCs (PVSMCs) were obtained and cultured in DMEM containing 10% FCS as described earlier (4). All experiments were performed between passages 2 and 5. Human aortic VSMCs (HVSMCs) were purchased from Clonetics (San Diego, CA) and cultured as per manufacturer’s instructions. In all the experiments VSMCs were serum depleted for 48 hours by transferring 80% confluent cells into medium containing 0.2% BSA prior to treatment with agonists for the indicated time periods and then processed for protein or RNA extraction. Wild-type CHO cells were cultured in DMEM-F12 medium containing
10% FCS. CHO cells stably overexpressing rat AT$_1_a$ receptor (CHO-AT$_1_a$) were grown in the same medium containing 0.4 mg/ml Geneticin.

**ELISA.** Serum depleted HVSMCs were treated with the indicated agonists and the culture supernatants collected at the indicated time periods to assay IL-6 levels using an ELISA kit (R&D Systems).

**Transient transfections and luciferase assays.** Transient transfections and luciferase assays were performed as described earlier (5) using Effectene and Lipofectamine 2000 for PVSM and CHO-AT$_1_a$ cells respectively. In some experiments, HVSMCs were electroporated (by nucleofection) using Amaza’s Nucleofector kit following manufacturer’s protocol and achieved transfection efficiency greater than 50%. Cells were serum depleted for 48 hours, stimulated with agonists for 6 hours, cell were lysed and luciferase assays performed according to manufacturer’s instructions.

**Immunoblotting.** Preparation of cell lysates and immunoblotting were carried out as described earlier (5).

**[^3H]-Leucine incorporation** into cells was determined as described earlier (5).

**Immunofluorescence.** Cells were seeded onto coverslips, grown to 60-70% confluence and then serum-starved for 48 hrs. After agonist treatment for the indicated time periods, cells were fixed with 3% paraformaldehyde, permeabilized in 0.05% Triton-X-100, blocked with 1% BSA in PBS and then treated with 1:50 dilutions of antibodies to p65 or human IL-18R$_\alpha$ for 1 hr at 37$^\circ$C. They were then treated with secondary antibodies tagged to rhodamine or fluorescein for 1 hr at 37$^\circ$C and then visualized using appropriate filters on an Olympus fluorescence microscope. Images were captured with Pixera 600 CL camera using Viewfinder V3.0 software and processed using Adobe Photoshop 7.0.
**RNA isolation and RT-PCR.** Isolation of total RNA from VSMC or aortas, RT-PCR using gene-specific primers paired with Quantum RNA 18S primers and quantitation of PCR fragments in agarose gels were performed as described earlier (5,6).

The primers used were: human IL-18Rα (sense: 5'-CTTCACATTCTTGCCCCCAAT-3’; antisense: 5’-GCAGCTGCATCCAGTTATGA-3’), IL-6 (sense: 5’-TCCTGCAGAAAAAGGCAAAG-3’; antisense: 5’-GCCCAGTGGACAGGTTTCT-3’), IL-8 (sense: 5’-AGGGTTGCCAGATGCAATAC-3’; antisense: 5’-GCAAACCCATTCAATTCCCTG-3’), MCP-1 (sense: CAAACTGAAGCTCGCACTC-3’; antisense: CATTTCACAATAATATTTTAG-3’), mouse IL-18Rα (sense: 5’-GTGCACAGGAATGAAACAGC-3’; antisense: 5’-ATTTAAGGTCCAATTGCACG-3’).

**REFERENCES:**


Online Materials and Methods:

Materials. Ang II was obtained from Peninsula laboratories (San Carlos, CA). Phospho-specific antibodies for STAT-1, STAT-3, p38, ERK, JNK/SAPK, AKT, Src, PKC α/β and antibody for total STAT-3 were from Cell Signaling (Beverly, MA). Anti-NF-kB p65 antibody was from Rockland Inc. (Gilbertsville, PA). IL-6 ELISA kit, recombinant human IL-18 and anti-human IL-18 Rα antibody were from R&D Systems (Minneapolis, MN). Restriction and modification enzymes were from New England Biolabs (Beverly, MA). Transfection reagent Lipofectamine 2000 and TA cloning kit were from Invitrogen (Carlsbad, CA). Nucleofector kit was from Amaxa Biosystems (Cologne, Germany). RNeasy columns, QIAshredder columns, and transfection reagent Effectene were from Qiagen (Valencia, CA). Reagents for immunofluorescence were from Molecular Probes (Eugene, OR). Quantum RNA 18S primers were from Ambion, Inc (Austin, TX) and cDNA kits from Applied Biosystems (Foster City, CA). Chromatin immunoprecipitation (ChIP) assay kit was from Upstate, Inc (Charlottesville, VA). Plasmid expressing dominant negative STAT-3 was a generous gift from Dr. Allan R. Brasier (University of Texas Medical Branch, Galveston) (1). The AP-1-Luc plasmid carrying three AP-1 binding sites upstream of the luciferase reporter gene was from Dr. Jian-Jian Li (Beckman Research Institute of City of Hope, Duarte). Promoterless pGL3b vector and luciferase assay reagents were from Promega (Madison, WI). CHO-AT1a cells (2,3) were a generous gift from Dr. Eric Clauser (INSERM EPI 0103, France).

Cell Culture and treatment with Ang II or IL-18. Primary cultures of porcine VSMCs (PVSMCs) were obtained and cultured in DMEM containing 10% FCS as described earlier (4). All experiments were performed between passages 2 and 5. Human aortic VSMCs (HVSMCs) were purchased from Clonetics (San Diego, CA) and cultured as per manufacturer’s instructions. In all the experiments VSMCs were serum depleted for 48 hours by transferring 80% confluent cells into medium containing 0.2% BSA prior to treatment with agonists for the indicated time periods and then processed for protein or RNA extraction. Wild-type CHO cells were cultured in DMEM-F12 medium containing
10% FCS. CHO cells stably overexpressing rat AT1a receptor (CHO-AT1a) were grown in the same medium containing 0.4 mg/ml Geneticin.

**ELISA.** Serum depleted HVSMCs were treated with the indicated agonists and the culture supernatants collected at the indicated time periods to assay IL-6 levels using an ELISA kit (R&D Systems).

**Transient transfections and luciferase assays.** Transient transfections and luciferase assays were performed as described earlier (5) using Effectene and Lipofectamine 2000 for PVSM and CHO-AT1a cells respectively. In some experiments, HVSMCs were electroporated (by nucleofection) using Amxaa’s Nucleofector kit following manufacturer’s protocol and achieved transfection efficiency greater than 50%. Cells were serum depleted for 48 hours, stimulated with agonists for 6 hours, cell were lysed and luciferase assays performed according to manufacturer’s instructions.

**Immunoblotting.** Preparation of cell lysates and immunoblotting were carried out as described earlier (5).

**[^3H]-Leucine incorporation** into cells was determined as described earlier (5).

**Immunofluorescence.** Cells were seeded onto coverslips, grown to 60-70% confluence and then serum-starved for 48 hrs. After agonist treatment for the indicated time periods, cells were fixed with 3% paraformaldehyde, permeabilized in 0.05% Triton-X-100, blocked with 1% BSA in PBS and then treated with 1:50 dilutions of antibodies to p65 or human IL-18Rα for 1 hr at 37°C. They were then treated with secondary antibodies tagged to rhodamine or fluorescein for 1 hr at 37°C and then visualized using appropriate filters on an Olympus fluorescence microscope. Images were captured with Pixera 600 CL camera using Viewfinder V3.0 software and processed using Adobe Photoshop 7.0.
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**REFERENCES:**


