STAT1 regulates interferon-γ-induced angiotensinogen and MCP-1 expression in a bidirectional manner in primary cultured mesangial cells

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Abstract

Objective: Intrarenal interferon-γ significantly contributes to the development of glomerular injury in which angiotensinogen and monocyte chemoattractant protein 1 levels are elevated. However, the exact nature of the role that interferon-γ plays in regulating angiotensinogen and monocyte chemoattractant protein 1 expression has not been fully delineated. Therefore, the aim of this study was to investigate the role that interferon-γ plays in angiotensinogen and monocyte chemoattractant protein 1 expression.

Methods: Primary cultured rat mesangial cells were treated with 0–20 ng/mL interferon-γ for 2, 8 or 24 hours. Expression levels of angiotensinogen, monocyte chemoattractant protein 1, suppressors of cytokine signaling 1, an intracellular suppressor of Janus kinase-signal transducers and activators of transcription signaling and activity of the Janus kinase-signal transducers and activators of transcription pathway were evaluated by reverse transcriptase polymerase chain reaction and western blot analysis.

Results: Interferon-γ increased angiotensinogen expression in mesangial cells with maximal augmentation observed following 5 ng/mL interferon-γ at 8 hours of treatment (1.87 ± 0.05, mRNA, relative ratio). Further increases were reduced or absent using higher concentrations of interferon-γ. Following treatments, monocyte chemoattractant protein 1 expression was induced in a linear dose-dependent manner (6.85 ± 0.62-fold by 20 ng/mL interferon-γ at 24 hours). In addition, interferon-γ induced STAT1 phosphorylation and suppressors of cytokine signaling 1 expression in a linear dose-dependent manner. The suppression of STAT1 and suppressors of cytokine signaling 1 expression by small interference RNAs facilitated an increase in interferon-γ-induced angiotensinogen expression, indicating that these two factors negatively regulate angiotensinogen expression. In contrast, the increase in interferon-γ-induced monocyte chemoattractant protein 1 expression was attenuated in STAT1-deficient mesangial cells, suggesting that STAT1 positively regulates monocyte chemoattractant protein 1 expression in mesangial cells.

Conclusion: These results demonstrate that while interferon-γ increases both angiotensinogen and monocyte chemoattractant protein 1 expression, STAT1 plays an opposing role in the regulation of each factor in mesangial cells.

Keywords

Interferon-γ, JAK-STAT pathway, angiotensinogen, MCP-1, mesangial cell

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Introduction

The renin–angiotensin system (RAS) plays important roles in blood pressure control through regulation of electrolyte and body fluid volume homeostasis.1 As intrarenal angiotensin II (Ang II) is elevated in many forms of renal injury and hypertension, intrarenal RAS is recognised as a key target for clinical and biological studies.2 It has been shown that activation of RAS is involved in the development of...
glomerular injury. Intrarenal angiotensinogen (AGT), the precursor of Ang II, is mainly expressed in renal proximal tubular cells; however, mesangial cells (MCs) in the glomerulus also produce AGT, thus providing a basis for the development of glomerular injury when AGT expression is left unchecked. RAS blockade has been shown to ameliorate the development of anti-glomerular basement membrane nephritis, as well as augmentation of glomerular AGT expression. Furthermore, increased glomerular AGT levels have been reported in pediatric IgA nephropathy patients. These findings suggest that glomerular RAS, in particular AGT stimulation, can play an important role in the development of glomerular injury.

Previous studies indicate that chronic Ang II infusions combined with a high salt diet create an inflammatory response leading to increased production of interferon-γ (IFN-γ) by activated T cells, natural killer cells and macrophages. IFN-γ augments monocyte chemoattractant protein 1 (MCP-1) expression and superoxide production in MCs, and is therefore regarded as one of the proinflammatory cytokines responsible for the development of glomerular injury. In contrast, the protective effects of IFN-γ on the development of renal injury have also been reported, but the mechanisms underlying these differential effects remain unclear.

The Janus kinase (JAK)-signal transducers and activators of transcription (STAT) pathway is a key mechanism mediating RAS and proinflammatory factors associated with renal injury. IFN-γ is a known activator of JAK and STAT1 and STAT3. In addition, activation of the JAK-STAT pathway by IFN-γ induces expression of suppressor of cytokine signaling (SOCS), which feeds back to regulate JAK-STAT signaling negatively. Although IFN-γ increases AGT expression via activation of STAT1 in human hepatocytes, STAT1 has been shown to repress AGT expression via SOCS1 augmentation in renal proximal tubular cells. These results support the hypothesis that IFN-γ acts to increase AGT and MCP-1 expression via activation of the JAK-STAT pathway in MCs. However, the exact mechanism behind IFN-γ-induced AGT and MCP-1 augmentation remains unclear. This study was performed to determine the effect of IFN-γ on AGT and MCP-1 expression in MCs, and to delineate the role that JAK-STAT signaling plays in the regulation of each factor.

**Materials and methods**

**Cell culture**

Rat MCs were isolated from intact renal glomeruli of male Sprague–Dawley rats (10–14 weeks old) as described previously. The isolated cells were identified by staining of Thy1, which is a marker of MCs (Supplemental Figure 1(a) and (b)). Cells were cultured in RPMI 1640 medium (Invitrogen) supplemented with 15% heat-inactivated fetal calf serum (Invitrogen) and were plated at a density of $1 \times 10^6$ cells per well in six-well plates. Prior to stimulation, the cells were serum-starved for 24 hours; 0–20 ng/mL rat recombinant IFN-γ (PeproTech) were added to medium containing 5% serum to treat MCs (0–24 hours). In the experiments, cells treated without IFN-γ (0 ng/mL) were used as control groups.

**Antibodies**

A rabbit anti-Thy-1 antibody was obtained from Dako. A rabbit anti-angiotensin-converting enzyme antibody and a
rabit anti-Ang II type 1 receptor antibody were purchased from Santa Cruz Biotechnology. A mouse anti-phospho-STAT1 (Tyr 701) antibody, a rabbit anti-phospho-STAT3 (Tyr 705) antibody, a rabbit anti-STAT1 antibody and a rabbit anti-STAT3 antibody were obtained from Cell Signaling Technology. A rabbit anti-AGT antibody and a rabbit anti-SOCS1 antibody were purchased from Abcam. IRDye-labeled anti-mouse IgG and anti-rabbit IgG antibodies were obtained from Li-Cor as secondary antibodies in western blot analysis.

**Quantitative real-time reverse transcriptase polymerase chain reaction**

Quantitative real-time reverse transcriptase polymerase chain reaction (qRT–PCR) was performed to evaluate AGT, MCP-1 and SOCS1 mRNA expression levels using the TaqMan PCR system. For total RNA isolation, treated cells were washed with 3 mL of phosphate-buffered saline (PBS). PBS was aspirated and total RNA was isolated from the cells using BIO-ROBOT EZ 1 (Qiagen). Subsequently, qRT–PCR was performed as previously described. All samples were analysed in triplicate, and the data were normalised based on expression levels of rat β-actin mRNA.

**Western blot analysis**

Phosphorylation levels of STAT1 and STAT3, and expression levels of AGT, STAT1, STAT3 and SOCS1 were detected using western blot analysis in order to elucidate participation of the transcriptional factors in AGT regulation by IFN-γ in MCs. The western blot analysis was previously described. Recent studies have demonstrated that unphosphorylated STATs levels in nuclei can be changed and also play important roles in IFN-induced pathophysiological events. The use of whole-cell lysates to detect unphosphorylated STATs and dissociation of unphosphorylated STATs in nuclei from these cell lysate samples is very difficult. Thus, data were normalised based on rat β-actin protein expression levels instead of total STATs levels.

**RNA interference**

The contributions of STAT1, SOCS1 and STAT3 to changes in AGT expression by IFN-γ treatment were examined using small interference RNA (siRNA) technology as previously described. In brief, MCs were plated on six-well plates with STAT1-siRNA (Ambion; sense sequence; 5’-CCUUUGUGGUGGAACGACATT-3’), SOCS1-siRNA (Ambion; sense sequence; 5’-CGAGCAUUCGUGGAACGACATT-3’), and STAT3-siRNA (Ambion; sense sequence; 5’-GCAGAGUUCAAGCACCUGATT-3’), respectively. Treatment with the transfection reagent (Lipofectamine 2000; Invitrogen) alone did not show a difference in basal SOCS1 and basal AGT and MCP-1 expression levels compared with negative control siRNA (Ambion)-transfected MCs; therefore, the transfection reagent alone (siRNA (-) group) was used as control groups in the these experiments. Cells were harvested after 48 hours transfection with siRNA to ensure suppression of STAT1 and SOCS1 protein using western blot analysis. At this time point, cells were treated with IFN-γ to evaluate the roles of STAT1, SOCS1 and STAT3 in the regulation of AGT and MCP-1 expression.

**Immunofluorescence staining**

MCs were cultured in four-well chambers (Lab-Tek). After treatment with 5 ng/mL IFN-γ for 8 hours, the cells were rinsed with PBS and then fixed for 20 minutes by 4% paraformaldehyde. After 4 minutes incubation with 0.2% Triton X-100, the blocking agent Image-iT FX signal enhancer (Invitrogen, CA, USA) was added to the chambers. The cells were incubated with antibodies against STAT1 and STAT3 for 3 hours. After washing with PBS, the cells were incubated with an Alexa Fluor 594-labeled secondary antibody. ProLong Gold antifade reagent with DAPI (Invitrogen) was used as a nuclear stain and a mount reagent. Localisation of the stained proteins was observed and photographed under a fluorescence microscope (Olympus BX51, Olympus Optical Co. Ltd.).

**Statistical analysis**

Data are expressed as means ± SE. The data were analysed using Student’s t-test, Welch’s t-test or one-way analysis of variance (ANOVA) followed by the post hoc Bonferroni/Dunn multiple comparison test. A value of \( P<0.05 \) was considered statistically significant.

**Results**

**Regulation of AGT expression by IFN-γ in MCs**

To study the effect of IFN-γ on AGT expression, primary MCs isolated from intact renal glomeruli of Sprague-Dawley rats were used. MCs were validated by staining for the MC marker Thy1 as well as for AGT, angiotensin-converting enzyme (ACE) and AT1R (Supplemental Figure 1(c)–(e)). MCs were treated with 0–20 ng/mL of IFN-γ for 2, 8 and 24 hours. While IFN-γ treatment at 2 hours revealed no expression changes (Figure 1(a), open column), treatment at 8 and 24 hours significantly increased AGT mRNA levels (Figure 1(a), grey column; dark grey column). The greatest induction of AGT mRNA expression was observed in MCs treated with 5 ng/mL IFN-γ at 8 hours (Figure 1(a), 1.87 ± 0.05, ratio to control). Augmentation of AGT protein by 2 ng/mL IFN-γ
treatment for 24 hours was also observed (Figure 1(b), 1.56 ± 0.09, ratio to control).

Regulation of MCP-1 expression by IFN-γ in MCs

To study the effect of IFN-γ on MCP-1 expression, MCs were treated with 0–20 ng/mL IFN-γ for 2, 8 and 24 hours. IFN-γ increased MCP-1 mRNA expression in a linear dose-dependent manner at 8 and 24 hours of treatments. In the tested IFN-γ doses, the highest induction of MCP-1 augmentation was observed by treatment with 20 ng/mL IFN-γ at 24 hours (Figure 2, 6.85 ± 0.62-fold, ratio to control). The levels of MCP-1 elevation were smaller at 8 hours than those at 24 hours, although the highest induction of AGT was detected by 5 ng/mL IFN-γ treatment at 8 hours.

Activation of STAT1 and STAT3 by IFN-γ in MCs

To elucidate the molecular mechanism underlying the regulation of AGT and MCP-1 expression by IFN-γ, STAT1 (Figure 3(a)) and STAT3 phosphorylation levels (Figure 3(b)) were determined following 8 hours of IFN-γ treatment. Western blot analysis revealed that treatment with IFN-γ induced phosphorylation of STAT1 in a linear dose-dependent manner (10.61 ± 0.49-fold by 20 ng/mL IFN-γ, ratio to control). Whereas the greatest induction of STAT1 phosphorylation was observed following treatment with 20 ng/mL IFN-γ, STAT3 activation was greatest following 5 ng/mL IFN-γ treatment. In addition, IFN-γ treatment induced translocalisation of STAT1 and STAT3 into nucleus in MCs (Figure 3(c) and (d)).

Contribution of STAT1 to the IFN-γ-induced AGT and MCP-1 augmentations

To elucidate further the mechanisms underlying the regulation of AGT and MCP-1 by IFN-γ, siRNA protein knockdown studies were employed. As 20 ng/mL IFN-γ treatment induced the greatest STAT1 phosphorylation (Figure 3(a)), this dose was used in the following sets of experiments. Treatment with STAT1-siRNA phosphorylation (Figure 4(a)), this dose was used in the following sets of experiments. Treatment with STAT1-siRNA suppressed basal STAT1 protein expression (Figure 4(a), 0.45 ± 0.001, ratio to siRNA (–) group). Treatment with siRNA (–) showed no effect on the expected AGT augmentation following treatment with IFN-γ for 8 hours (Figure 4(b), 1.40 ± 0.03, ratio to control). The suppression of STAT1 by STAT1-siRNA resulted in further increases in AGT expression following IFN-γ treatment (Figure 4(b), 2.11 ± 0.08, ratio to control). In contrast, the increase in IFN-γ-induced MCP-1 augmentation was significantly attenuated in STAT1-deficient MCs (Figure 4(c)), suggesting that STAT1 positively regulates MCP-1 expression in MCs.

Regulation of SOCS1 expression by IFN-γ in MCs

As STAT1 knockdown led to the enhancement of AGT upregulation by IFN-γ, we hypothesised that the STAT1–SOCS1 axis serves as a suppressing mechanism in IFN-γ-induced AGT regulation as previously shown in renal proximal tubular cells. Therefore, SOCS1 regulation by IFN-γ was investigated in MCs. At 2 (Figure 5(a), open column), 8 (grey column) and 24 hours (dark grey column), IFN-γ treatment increased SOCS1 mRNA expression in a linear dose-dependent manner, albeit, to varying degrees. While treatment with 20 ng/mL IFN-γ at 2 hours led to the greatest induction of SOCS1 expression (438.8 ± 24.1, ratio to control), IFN-γ also induced significant levels of SOCS1 mRNA expression at both 8 hours (117.9 ± 5.2, ratio to control) and 24 hours (152.4 ± 2.58, ratio to control). Elevation of SOCS1 protein was also confirmed by western blot analysis (Figure 5(b)).

Participation of SOCS1 in the IFN-γ-induced AGT augmentation

To investigate whether STAT1 regulates SOCS1 augmentation, STAT1-knockdown studies were employed (Figure 6(a)). In the siRNA (–) group, IFN-γ treatment induced SOCS1 expression at 8 hours (Figure 6(a), 49.2 ± 1.9,
ratio to control). However, augmentation of SOCS1 expression was attenuated following STAT1-knockdown (Figure 6(a)), suggesting that STAT1 mediates the SOCS1 elevation.

SOCS1 expression was reduced by SOCS1-siRNA (Figure 6(b), 0.55 ± 0.09, ratio to siRNA (–) group) to demonstrate the participation of SOCS1 in IFN-γ-induced AGT augmentations. Knockdown of SOCS1 expression revealed results similar to those seen in the STAT1-siRNA studies. Treatment of SOCS1-siRNA MCs with IFN-γ resulted in further augmentation of AGT expression (Figure 6(c), 2.64 ± 0.10, ratio to control) when compared with control-siRNA treated cells (Figure 6(c), 1.32 ± 0.12, ratio to control). These results indicate that the STAT1–SOCS1 axis suppresses IFN-γ-induced AGT augmentation.

**Regulation of the IFN-γ-induced AGT augmentation by STAT3**

Whether STAT3 is involved in the IFN-γ-induced AGT regulation in MCs was tested using STAT3-siRNA. Basal STAT3 expression levels were decreased by STAT3 knockdown (Figure 7(a), 0.54 ± 0.03, ratio to siRNA (–) group). IFN-γ treatment stimulated AGT expression (Figure 7(b), 1.40 ± 0.03, ratio to control); however, the reduction of STAT3 by siRNA antagonised the augmentation of AGT expression by IFN-γ (Figure 7(b), 1.10 ± 0.03, ratio to control).
Discussion

Activation of the immune system is a crucial mechanism contributing to the progression of hypertension and associated tissue injury. Various studies have revealed that RAS and proinflammatory cytokines interact to elicit the development of hypertension and renal dysfunction. Activation of intrarenal RAS increases intrarenal IFN-γ levels, which leads to the development of glomerular injury. Importantly, AGT has been shown to be a key player in glomerular RAS activation and subsequent glomerular damage characterised by increases in MCP-1 expression. IFN-γ stimulates AGT expression via activation of the JAK-STAT pathway in renal proximal tubular cells and hepatocytes. However, AGT regulation by IFN-γ in the glomerulus, especially in MCs, has not been fully delineated. Here, we investigated IFN-γ-induced AGT regulation in MCs, and delineated the role that JAK-STAT signaling and SOCS1 play in this pathway.
Although shorter treatments of IFN-γ did not affect AGT levels in MCs, prolonged exposure to the cytokine increased AGT expression. Maximum AGT augmentation was induced by 5 ng/ml IFN-γ at 8 hours, whereas higher concentrations of IFN-γ induced smaller degrees of AGT expression. On the other hand, it has been demonstrated in rat renal proximal tubular cells that shorter treatment duration (6 and 12 hours) with 2–20 ng/ml IFN-γ lower AGT expression levels compared with baseline. These suppressing effects on AGT by higher concentrations of IFN-γ were not observed at earlier time points in MCs, suggesting that while AGT is augmented in both MCs and proximal tubular cells, the timing and concentration of maximal IFN-γ-induced AGT expression is occurring by different mechanisms that are most likely specific to each cell type.

IFN-γ induced activation of STAT1 and SOCS1 in a linear dose-dependent manner, suggesting that overactivation of the STAT1–SOCS1 axis by higher doses of IFN-γ antagonises AGT upregulation in MCs. Suppression of STAT1 and SOCS1 by siRNAs enhanced the IFN-γ-induced AGT upregulation, indicating that both factors are critical in the negative regulation of AGT expression. While IFN-γ expression.
stabilization of AGT expression, it also leads to activation of a STAT1–SOCS1 axis, which acts to limit the magnitude of AGT augmentation. These results demonstrate that while IFN-γ increases both AGT and MCP-1 expression, STAT1 plays an opposing role in the regulation of each factor in MCs. MCP-1: monocyte chemoattractant protein 1; AGT: angiotensinogen; IFN-γ: interferon-γ; SOCS1: suppressor of cytokine signaling 1; MCs: mesangial cells.

Figure 8. Schematic summary of proposed MCP-1, AGT regulation by IFN-γ-STAT1 in rat MCs. IFN-γ induces MCP-1 and SOCS1 expression directly via STAT1 in rat MCs. Conversely, STAT1-induced expression of SOCS1 attenuates IFN-γ-STAT3-mediated expression of AGT, providing feedback regulation of IFN-γ-AGT signaling in rat MCs. These results demonstrate that while IFN-γ increases both AGT and MCP-1 expression, STAT1 plays an opposing role in the regulation of each factor in MCs. MCP-1: monocyte chemoattractant protein 1; AGT: angiotensinogen; IFN-γ: interferon-γ; SOCS1: suppressor of cytokine signaling 1; MCs: mesangial cells.

The present study furthers our understanding of the relationship between IFN-γ-induced STAT3 activation and AGT expression. IFN-γ-induced STAT3 activation corresponds with increases in AGT expression. Furthermore, the reduction of STAT3 by siRNA abolished the augmentation of AGT expression by IFN-γ. These data agree with previous reports that demonstrated STAT3 binding to the rat AGT gene promoter with high affinity in Ang II-treated myocytes. Furthermore, previous studies have shown that loss of SOCS1 activity mediates increased STAT3 activity, indicating SOCS1 as a negative regulator of STAT3.27, 45 Our previous study using renal cells also indicated elevated SOCS1 by IFN-γ attenuates STAT3 activity.27 Accordingly, the STAT1–SOCS1 axis limits AGT expression elevation via down-regulation of STAT3 activity in MCs. The counteracting actions of STAT1 and STAT3 on AGT regulation in MCs are supported by findings in AGT regulation in renal proximal tubular cells27 and in other physiological phenomena such as cell apoptosis.46, 47 Thus, the balance of these transcription factors may be of importance for intrarenal AGT regulation and the establishment of kidney injury.

IFN-γ augments MCP-1 expression in MCs, which facilitates the development of glomerular injury.12–15 In the present study, IFN-γ treatment increased MCP-1 expression in a linear dose-dependent manner. As already mentioned, IFN-γ-induced AGT expression was enhanced by STAT1 knockdown. In contrast, the STAT1 knockdown attenuated IFN-γ-induced MCP-1 upregulation, indicating that activated STAT1 mediates MCP-1 expression. This is opposite to the repressive role that STAT1 plays in IFN-γ-induced AGT regulation. These findings suggest that while IFN-γ increases both AGT and MCP-1 expression, STAT1 plays an opposing role in the regulation of each factor (Figure 8) in mesangial cells.

While it is clear that JAK-STAT signaling is involved in both IFN-γ-induced AGT and MCP-1 expression, the results obtained from the present study suggest that the mechanism underlying IFN-γ-induced AGT augmentation is different from the mechanism underlying MCP-1 regulation in MCs. These findings provide a novel and more effective approach to treat glomerular diseases and dysfunction by targeting aberrant activation of the intrarenal JAK-STAT pathway.

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