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Interferon-γ enhances superoxide production in human mesangial cells via the JAK–STAT pathway

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Immune reactive cytokines, such as interferon (IFN)-γ, have multiple effects in glomerulonephritis. Superoxide anions (O_2^-), which are associated with the progression of glomerulonephritis, are mainly generated by nicotinamide adenine dinucleotide phosphate (reduced form) NAD(P)H oxidases. We determined the effects of IFN-γ on O_2^- production, phosphorylation of signal transducer and activator of transcription (STAT)-1α, and the mRNA and protein expressions of p22phox and Nox1, components of NAD(P)H oxidases, in human mesangial cells (HMCs). Significant increases in O_2^- production with IFN-γ were completely abolished by the flavin-containing enzyme inhibitor, diphenyleneiodonium (10 μmol/l), and the Janus-activated kinase (JAK)2 inhibitor, AG490 (100 μmol/l). Phosphorylated STAT-1α was detected after 5 min of IFN-γ stimulation using Western blot analysis, and binding to the gamma-activating site was observed from 30 min to 4 h, thereafter by electrophoretic mobility shift assay (EMSA). Super-shift analysis in EMSA revealed that the main transcription factor was STAT-1α. IFN-γ significantly increased the expression of p22phox mRNA and protein, although expression was inhibited by AG490. These data suggest that IFN-γ stimulates O_2^- production in HMCs via the JAK–STAT pathway and NAD(P)H oxidase.

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Interferon (IFN)-γ, a proinflammatory factor produced by T helper 1 cells, mediates biological actions such as macrophage activation, antiviral activity, and production of free radicals.[1] Previous reports have indicated that IFN-γ is associated with kidney inflammation including nephritis and arteriosclerosis.[2,3] Serum levels and intrarenal expression of IFN-γ increases in human nephritis,[4,5] and IFN-γ gene polymorphisms influence disease susceptibility and progression in human chronic glomerulonephritis.[6] In *in vitro* experiments, the signal-transduction pathway of IFN-γ has been elucidated in immune-responsive cells. IFN-γ usually binds to a specific membrane receptor and activates receptor-associated Janus-activated kinase (JAK)1 and JAK2.[1] JAK1 and JAK2 activate the signal transducer and activator of transcription-1α (STAT-1α) with phosphorylation of the tyrosine residue, which forms homodimers that translocate to the nucleus. This induces transcription via binding to the gamma-activating site (GAS) in IFN-γ-inducible promoters and provokes the production of various proteins.[1] These molecular mechanisms of IFN-γ signal-transduction systems have been found in immune-responsive cells; recently, the mechanism has also been reported in glomerular mesangial cells. Choudhury[7] reported that IFN-γ stimulates protein kinase C-ε in a phosphatidylinositol 3-kinase-sensitive manner to activate the mitogen-activated protein kinase that regulates STAT-1α transcriptional activity. However, the molecular mechanisms by which IFN-γ progresses renal injury have not been elucidated.

We previously reported that reactive oxygen species (ROS) have important roles in the pathogenesis of diseases such as arteriosclerosis and hypertension.[8,9] Enhanced ROS production has also been reported in human and experimental glomerulonephritis.[10] Infiltrated polymorphonuclear leukocytes and monocytes are recognized as an important source of ROS in the kidney, and resident glomerular cells, such as mesangial cells, also release oxidants in response to injury.[11] Further, one of the major sources of superoxide (O_2^-) in the kidney is nicotinamide adenine dinucleotide phosphate (reduced form) (NAD(P)H) oxidase in mesangial cells.[12] Recent studies have shown that three isoforms of the components of NAD(P)H oxidase, Nox1, gp91phox, and

Nox4 are found in the kidney.[13] Jones *et al.*[12] reported that the other NAD(P)H oxidase components, p22phox, p47phox, and p67phox, are expressed in cultured human mesangial cells (HMCs). Circulating vasoactive peptides and inflammatory cytokines including IFN-γ also stimulate ROS production in nephropathy.[2,14] However, the molecular and cell signaling mechanisms of ROS production by IFN-γ have not been clarified.

We hypothesized that IFN-γ, which is induced under inflammatory conditions, regulates NAD(P)H oxidase components and that IFN-γ is a candidate for ROS-associated glomerular damage in human nephritis. To ascertain the molecular mechanisms and the signaling pathway of IFN-γ in cultured HMCs, we investigated the effects of IFN-γ on the JAK–STAT pathway, the expression of NAD(P)H oxidase components and ROS production.

RESULTS

Effects of IFN-γ on STAT phosphorylation

To clarify the mechanism underlying IFN-γ-induced transcription in HMCs, we performed Western blot analysis and electrophoretic mobility shift assay (EMSA). As shown in Figure 1a, 5-min exposure to IFN-γ increased the phosphorylation of STAT-1α by 1.8-fold relative to the unstimulated control. Hence, IFN-γ did not alter the expression of STAT-1α protein at any time (Figure 1a, lower picture), and indicates that IFN-γ phosphorylated STAT-1α in cultured HMCs. The STAT-1α phosphorylation after 5-min incubation with IFN-γ was partially inhibited by AG490 (100 μmol/l), a JAK2 inhibitor (Figure 1b). Diphenyleneiodonium (DPI) did not affect the STAT-1α phosphorylation (Figure 1c). These data indicate that IFN-γ phosphorylates STAT-1α via the JAK–STAT pathway.

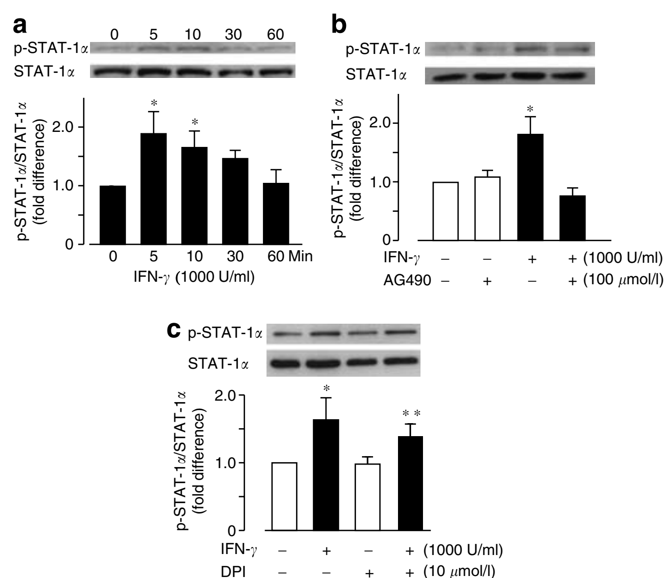

Figure 1 | Effect of IFN-γ on the phosphorylation of STAT-1α in HMCs. (**a**) Western blot analysis using phosphorylated STAT-1α (p-STAT-1α) and STAT-1α antibody (upper and lower pictures, respectively) shows that IFN-γ increased phosphorylation of STAT-1α within 5 min, but did not change STAT-1α protein levels. Values represent the mean ± s.e. of four independent experiments. *$P<0.01$ vs control. (**b**) Western blot analysis using p-STAT-1α and STAT-1α antibody (upper and lower pictures, respectively) shows that the JAK2 inhibitor AG490 inhibited the IFN-γ-induced phosphorylation of STAT-1α at 5 min. Values represent the mean ± s.e. of four independent experiments. *$P<0.01$ vs control. (**c**) Western blot analysis using p-STAT-1α and STAT-1α antibody (upper and lower pictures, respectively) shows that flavin-containing enzyme inhibitor DPI inhibited the IFN-γ-induced phosphorylation of STAT-1α at 5 min. Values represent the mean ± s.e. of four independent experiments. *$P<0.05$ vs unstimulated control. **$P<0.05$ vs unstimulated control with DPI.

We then performed EMSA using the GAS element as the radiolabeled oligonucleotide probe. Figure 2a shows that IFN-γ promoted binding of nuclear extracts to the GAS sequence of HMCs. There was binding activity to the GAS element after 5-min treatment with IFN-γ. Binding activity peaked at 30 min and was retained for 4 h. The specificity of IFN-γ-promoted binding was confirmed by competitively inhibiting this reaction with a 50-fold excess of non-labeled GAS oligonucleotide. We also measured the effect of the antibody to STAT-1α on the migration of the DNA–protein complex by EMSA. In Figure 2b, the broken arrow in lane 3 indicates the 'super-shifted' band induced by the STAT-1α antibody (8 μg/ml). The DNA–protein complex associated with the GAS element was super-shifted by pre-incubation with the STAT-1α antibody, indicating that the complex consists of STAT-1α. These data show that IFN-γ activates STAT-1α, and that activated STAT-1α binds to the GAS sequence in cultured HMCs.

Effects of IFN-γ on the expression of p22phox and Nox1

Using Northern blot analysis, we investigated the time course of p22phox mRNA expression in cultured HMCs treated with IFN-γ; we detected p22phox mRNA (Figure 3a) and found that IFN-γ significantly upregulated its expression from 3 to 24 h relative to glyceraldehyde-3-phosphate dehydrogenase (Figure 3, lower panel). We also detected Nox1 mRNA; however, IFN-γ did not increase the expression of Nox1 mRNA (Figure 3b). We measured the protein expression of p22phox using Western blot analysis. IFN-γ significantly increased p22phox protein at 6, 12, and 24 h relative to β-actin (Figure 4a). The JAK2 inhibitor AG490 (100 μmol/l) completely inhibited the increase of p22phox induced by IFN-γ (Figure 4b), whereas DPI did not affect p22phox protein expression (Figure 4c). These data indicate that IFN-γ increases p22phox mRNA and protein expression via the JAK–STAT pathway.

Effects of IFN-γ on O_2^- production

Using lucigenin-enhanced chemiluminescence, we analyzed the time course of O_2^- production in HMCs treated with IFN-γ (1000 IU/ml). As shown in Figure 5a, IFN-γ gradually increased the amount of O_2^-, with significant two- and 2.8-fold increases at 12 and 24 h, respectively. To confirm the involvement of NAD(P)H oxidase, we examined the effect of DPI, a flavin-containing enzyme inhibitor. The IFN-γ-mediated increase in the amount of O_2^- at 24 h was

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