Molecular mechanisms of iNOS induction by IL-1β and IFN-γ in rat aortic smooth muscle cells

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Teng, Xingwu, Hanfang Zhang, Connie Snead, and John D. Catravas. Molecular mechanisms of iNOS induction by IL-1β and IFN-γ in rat aortic smooth muscle cells. Am J Physiol Cell Physiol 282: C144–C152, 2002.—In rat aortic smooth muscle cells (RASMC), interferon (IFN)-γ enhanced nitrite accumulation and type II nitric oxide synthase (iNOS) protein expression induced by interleukin (IL)-1β. IFN-γ alone had no effect on nitrite accumulation or iNOS protein. IL-1β, but not IFN-γ, induced nuclear factor (NF)-κB and CCAAT box/enhancer binding protein (C/EBP) nuclear binding. Conversely, IFN-γ, but not IL-1β, induced signal transducer and activator of transcription (STAT) 1 and interferon regulatory factor (IRF)-1 nuclear binding. In a ~1.4-kb rat iNOS promoter segment, deletion of an IFN-γ-activated site (GAS) increased IL-1β-induced activity but inhibited IFN-γ-enhanced activity, suggesting a two-way effect of the GAS site on iNOS induction: enhancing induction through STAT1 activation and inhibiting induction through a non-IFN-γ-mediated mechanism. Deletion of both an IRF and a C/EBP site reduced the IL-1β-induced and the IFN-γ-enhanced activities. However, IRF site mutations decreased the IFN-γ-enhanced activity without affecting the IL-1β-induced activity. Insertion of two IRF sites increased the IFN-γ-enhanced activity without affecting the IL-1β-induced activity. Insertion of two IRF sites increased the IFN-γ-enhanced activity without affecting the IL-1β-induced activity. Insertion of two IRF sites increased the IFN-γ-enhanced activity without affecting the IL-1β-induced activity. Insertion of two IRF sites increased the IFN-γ-enhanced activity without affecting the IL-1β-induced activity.

NITRIC OXIDE (NO), a multifunctional effector molecule synthesized by nitric oxide synthase (NOS) from l-arginine (28), transmits signals for vasorelaxation, neurotransmission, and cytotoxicity. Three different NOS isoforms have been identified, which fall into two distinct types, constitutive and inducible. Unlike the constitutively expressed NOS isoforms (eNOS, or type III NOS; nNOS, or type I NOS) (4, 29), the activity of inducible NOS (iNOS, or type II NOS) isoform is independent of intracellular calcium concentration and yields much higher NO output (26, 35). The excess NO produced by iNOS is implicated in the pathophysiological processes of cardiovascular diseases such as sepsis (17), stroke (2), and atherosclerosis (7). However, the mechanisms underlying iNOS expression in vascular diseases remain unclear. Unraveling the molecular mechanisms responsible for iNOS induction may shed light on ways to manage these diseases. Interferon (IFN)-γ alone is reported to induce iNOS expression in cultured human and rat vascular smooth muscle cells (SMC) (6, 19) but not in cultured rat pulmonary artery SMC (27), aortic SMC (1), and the SMC cell line A7r5 (33). Similarly, interleukin (IL)-1β alone has been shown to induce iNOS expression in cultured rat aortic SMC (1, 11, 16, 19) but not in cultured human vascular SMC (6), rat pulmonary artery SMC (27), and the SMC cell line A7r5 (33). Variations in species and cell types and in long-term cultured cell line vs. short-term cultures from primaries may account for the observed different effects. Thus, in each experimental model, the effect of IFN-γ or IL-1β on iNOS induction must be clarified before the molecular mechanisms of iNOS induction can be investigated.

Previous studies have shown that nuclear factor (NF)-κB sites are required for iNOS induction (12, 33, 36, 37, 39). The importance of NF-κB sites also was confirmed by NF-κB inhibition (14). Besides NF-κB, other transcription factors, such as interferon regulatory factor (IRF)-1, CCAAT box/enhancer binding protein (C/EBP), and signal transducer and activator of transcription (STAT) 1, also are involved in iNOS induction. IRF-1 is required for iNOS induction in mice, as demonstrated by the IRF-1 gene knockout (15). Mutation of the IRF-1 binding site in murine iNOS promoter showed that the site mediates the IFN-γ-enhanced iNOS promoter activity (24). STAT1α induction and its binding to the interferon-γ activation site (GAS) in murine iNOS promoter mediates iNOS promoter induction by IFN-γ and lipopolysaccharide (LPS) in RAW 264.7 cells (13). C/EBP induction and binding to a C/EBP site in rat iNOS promoter mediates the iNOS promoter induction by cAMP in rat mesangial cells (12).

The mechanisms of iNOS induction are cell and species specific. For full promoter activity, different lengths of the 5′ flanking regions among murine, rat,
and human iNOS promoters are required. The 1 kb of murine iNOS promoter confers full promoter activity (22). To confer full promoter activity in the rat, 2 kb of additional 5′ flanking region are required (40). The first 3 kb of human iNOS promoter exhibits no activity, and over 10 kb of the 5′ flanking region are required for full human iNOS promoter activity (10, 21). Similarly, iNOS induction is cell specific. The Janus kinase (JAK)/STAT pathway mediates the LPS plus IFN-γ-induced iNOS expression in RAW 264.7 cells (13). However, inhibition of JAK/STAT pathway enhances iNOS induction by LPS plus IFN-γ in rat aortic smooth muscle cells (RASM) (23).

We recently cloned the rat iNOS promoter (40). Because of the differences in iNOS induction among species, we have used a homologous system, rat iNOS promoter transfected into rat SMC, to eliminate possible species differences in the regulation of iNOS. We now report that in RASM, IL-1β alone, but not IFN-γ alone, induces iNOS. However, IFN-γ enhances iNOS induction by IL-1β. We further present data showing that the IL-1β-induced iNOS expression is mediated by NF-κB and C/EBP activation and that IFN-γ-enhanced promoter activity is mediated by IRF-1 and STAT1 activation.

MATERIALS AND METHODS

Chemicals. Rat recombinant IFN-γ was obtained from R&D Systems (Minneapolis, MN), and (human) IL-1β was purchased from Boehringer Mannheim (Indianapolis, IN). The concentrations of cytokines used in the study were similar to those used by other investigators in vascular SMC (6, 33). Lipofectamine was purchased from Life Technologies (Gaithersburg, MD). Gel shift antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA).

Cell cultures. RASM were harvested from Wistar rats weighing 200–225 g (Harlan, Indianapolis, IN) by enzymatic dissociation. The cells were positively identified as smooth muscle by indirect immunofluorescent staining for α-actin with the use of mouse anti-α-actin antibody and anti-mouse IgG-FITC conjugate. Cells were grown in 50% Dulbecco’s modified Eagle’s medium and 50% F-12 nutrient medium (DMEM/F-12) supplemented with 10% fetal bovine serum, 200 IU/ml of penicillin, 200 μg/ml of streptomycin, 2 mM glutamine (0.2 g/l), penicillin (100 units/ml), and streptomycin (100 units/ml). All cultures were grown in a humidified atmosphere at 37°C with 5% CO2 in air. Cells at passages 2–4 were used in the studies.

Nitrite assay. Nitrite levels were measured by a fluorometric method (25). First, 150 μl of cell culture supernatant were diluted in 2 ml of H2O and mixed with 200 μl of 2,3-diaminonaphthalene (0.05 mg/ml 0.62 M HCl). After incubation at room temperature for 15 min, 100 μl of NaOH (2.8 M) were added to stop the reaction. The fluorescence intensity was measured at 380 nm (excitation) and 407 nm (emission) with a Spectrofluorometer (Perkin-Elmer 5B).

Western blot. After the experiment protocol, RASM were harvested twice with cold PBS and 300 μl of the lysis buffer [20 mM Tris-HCl, pH 7.4, 2.5 mM EDTA, 1% Triton, 10% glycerol, 1% deoxycholate, 0.1% SDS, 50 mM NaF, 10 mM Na3VO4, 1 mM Na2P2O7, and 1 mM phenylmethylsulfonyl fluoride (PMSF)] were added. The lysates containing an equal amount of protein (10 μg) were subsequently loaded on 7.5% SDS polyacrylamide gels, and the resolved proteins were electrophoretically transferred to nitrocellulose membrane. iNOS protein was specifically detected by rabbit polyclonal anti-mouse iNOS antibody with 1: 5,000 dilution (Transduction Laboratories, Lexington, KY). The second antibody was a peroxidase-conjugated anti-rabbit IgG from donkey. Membrane was developed with the enhanced chemiluminescence detection system (ECL; Amersham, Piscataway, NJ) and exposed on film.

Nuclear extracts. The nuclear protein isolation protocol was modified from the method of Sadowski and Gilman (31). After treatment, RASM were rinsed with ice-cold PBS and hypotonic buffer [20 mM HEPES-KOH, pH 7.9, 20 mM NaF, 1 mM Na3VO4, 1 mM Na2P2O7, 0.4 μM microcystin, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol (DTT), 1 mM PMSF, and 1 μg/ml each of leupeptin, aprotinin, and pepstatin]. Ice-cold hypotonic buffer (0.5 ml) with 0.5% Nonidet P-40 was added to the dish (100 mm) and incubated on ice for 10 min. The lysates were scraped into a glass homogenizer and stroked 12 times. The homogenate was transferred to a 1.5-ml tube and vortexed vigorously for 10 s. The nuclei were pelleted by centrifugation at 12,000 rpm for 2 min at 4°C, washed with 500 μl of hypotonic buffer once, resuspended in 120 μl of high-salt buffer (hypotonic buffer plus 420 mM NaCl and 20% glycerol), and then rocked gently for 30 min at 4°C. The extracted nuclear proteins were separated from residual nuclei by centrifugation at 12,000 rpm at 4°C for 30 min. The supernatant (nuclear proteins) was collected and frozen at −80°C. Protein concentration in nuclear extracts was measured with the Bradford assay (3).

Electrophoretic mobility shift assays. The DNA probes were hybridized and end-labeled with T4 polynucleotide kinase in a reaction containing 2 μl of probe (1.75 pmol/μl), 1 μl of 10× T4 kinase buffer, 1 μl of [γ-32P]ATP (10 μCi/ml), 5 μl of water, and 1 μl of T4 kinase (10 units/μl). After incubation at 37°C for 30 min, the reaction was stopped by adding 1 μl of 0.5 M EDTA and then 89 μl of Tris base-EDTA buffer. The labeled probe was separated from unincorporated [γ-32P]ATP chromatographically on G6 spin columns (Bio-Rad, Hercules, CA).

For each binding reaction, aliquots of the nuclear extract (5 μg) were suspended in a final volume of 12.5 μl by adding 2.5 μl of 5× binding buffer [100 mM HEPES-KOH, pH 7.9, 250 mM KCl, 5 mM MgCl2, 5 mM DTT, 0.5 mM EDTA, 1 mg/ml bovine serum albumin, 0.5 μg/ml poly(dI-dC), and 25% glycerol], an appropriate amount of water, and 1 μl of radiolabeled probe. Before the labeled probe was added, the mixture was incubated on ice for 10 min. After the probe was added, the incubation continued at room temperature for 20–25 min. For supershift, 1 μl of antibody was included in the 12.5-μl binding reaction volume, and it was added 10 min before the labeled probe was added. Reactions were analyzed by electrophoresis on a 4% nondenatured polyacrylamide gel in 0.5× Tris-boric acid-EDTA at 200 V and 4°C. Gels were dried, and bands corresponding to complexes between transcription factors and the labeled probe were detected by autoradiography.

Plasmids. The construction of a ~3.2-kb rat iNOS promoter-luciferase construct has been described previously (40). The −1.4-kb rat iNOS promoter-luciferase construct was generated by using the restriction enzymes SphI and XhoI to cut the DNA from the −3.2-kb rat iNOS promoter-pGL3basic construct and was ligated with the pGL3basic vector. Sequencing confirmed the 5′ end of promoter located at −1368 bp. To generate the mutated rat iNOS promoter constructs, we performed site-directed mutagenesis on the context of the −1.4-kb reporter construct, using the QuickChange site-directed mutagenesis as follows. The −1.4-kb rat iNOS promoter reporter construct was used as the template. Two
Table 1. Primers for mutagenesis experiments

<table>
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<th>Rat iNOS-promoter sequence</th>
<th>C/EBP(8/9)</th>
<th>NF-κB(10/12)</th>
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<tr>
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</tr>
<tr>
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<td>cccctctctctgtttgttcctt tactgtcaatatttcac</td>
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</tr>
<tr>
<td>IRF-Mu3</td>
<td>ccccttaactgtcaatatatttcactttcataatggaaaatccc</td>
<td>cccctctctctgtttgttcctt tactgtcaatatttcac</td>
</tr>
</tbody>
</table>

Underlined characters represent mutated nucleotide; italicized characters represent inserted (Ins) nucleotides; spaces in the sequences are where the deletions (Del) occur. Only the lead strands are shown at 5’ to 3’.

RESULTS

Role of a GAS site in rat iNOS promoter induction.

With the use of a rat iNOS promoter fragment (−941 to −922 bp) containing a GAS site (−936 to −928 bp) as a probe (Fig. 3A), electrophoretic mobility shift assays (EMSA) showed that IFN-γ, but not IL-1β, induced a factor binding to the probe; this factor was identified as STAT1 from the EMSA band supershifted by anti-STAT1α antibody (Fig. 3B). Deletion of the GAS site increased the IL-1β-induced promoter activity but decreased the IFN-γ-enhanced promoter activity (Fig. 3, and Table 1).

IFN-γ enhances IL-1β-induced iNOS expression in RASMC. RASMC were treated with or without IFN-γ for 24 h. After treatment, the medium was collected and the concentration of nitrite, the stable metabolite of NO, was measured. As shown in Fig. 1, IFN-γ (recombinant, 250 units/ml) alone could not induce nitrite accumulation in RASMC; IL-1β alone strongly induced nitrite accumulation (121.4 ± 12.0-fold higher than control). IFN-γ significantly enhanced the nitrite accumulation induced by IL-1β (from 121.4 ± 12.0- to 177.6 ± 11.4-fold higher than control, P < 0.01) or IL-1β plus tumor necrosis factor (TNF)-α (from 157.3 ± 9.8- to 223.8 ± 14.0-fold higher than control, P < 0.01) (Fig. 1). Similarly, IFN-γ alone did not induce iNOS protein but enhanced IL-1β-induced iNOS protein expression (Fig. 2).

Fig. 1. Nitrite accumulation in rat aortic smooth muscle cells (RASMC). Confluent RASMC (passage 3 to 4) were treated with 100 units/ml interleukin-1β (IL-1), 250 units/ml rat recombinant interferon-γ (IFN), or a combination of IL-1, IFN, and tumor necrosis factor-α (TNF; 550 units/ml) in DMEM/F-12 containing 10% fetal bovine serum for 24 h. The medium was then collected, and nitrite concentration was measured, normalized to the control group, and expressed as a multiple of control. Nitrite concentration in the control group was 364 ± 23 nM. Data are from at least 2 experiments (n = 6–17), each performed in triplicate, and are expressed as means ± SE. Student’s t-test or one-way ANOVA, followed by the least significant different procedure, as appropriate, and differences at P < 0.05 were considered statistically significant.

RESULTS

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C and D). These results suggest a two-way effect of the GAS site on iNOS induction: an enhancement mediated by an IFN-γ-dependent mechanism, presumably JAK/STAT pathway, and an inhibition mediated by an unknown IFN-γ-independent mechanism.

Role of IRF and C/EBP sites in IFN-γ rat iNOS promoter activation. With the use of a rat iNOS promoter fragment (−921 to −898 bp) containing an IRF (−918 to −907 bp) and a C/EBP (−910 to −902 bp) site as probe (Fig. 4A), EMSA showed that IFN-γ, but not IL-1β, induced IRF-1 binding to the probe and that the binding disappeared in the presence of anti-IRF-1 antibody (Fig. 4B). Conversely, IL-1β, but not IFN-γ, induced a C/EBP binding to the probe, and IFN-γ did not enhance the IL-1β-induced C/EBP binding. The C/EBP binding band disappeared in the presence of anti-C/EBP antibody (Fig. 4C). However, the IRF-1 and C/EBP bands were not significantly changed in the presence of either anti-p50 or anti-p65 antibodies (Fig. 4D). Deletion mutation (Del-IRF-C/EBP) of −914 to −905 bp, which disrupted both the IRF and the C/EBP sites, abolished both the IL-1β-induced and IFN-γ-enhanced promoter activities (Fig. 5A). To distinguish between the roles of the IRF and the C/EBP sites, three loss-of-function mutations of the IRF site were generated outside of the C/EBP motif to disrupt only the IRF site. One mutation consisted of an insertion of an XbaI site (IRF-Ins6) between −911 and −910 bp. The other two were substitution mutations; one substituted AGT for TCA at −914 to −912 bp (IRF-Mu3), and the other substituted GG for TT at −916 to −915 bp (IRF-Mu2).

Fig. 2. IFN enhances type II nitric oxide synthase (iNOS) protein expression by IL-1 in RASMC. Passage 3 confluent RASMC were treated with 100 units/ml IL-1, 250 units/ml rat recombinant IFN, or both for 24 h. The cells were then lysed in RIPA buffer, and 10 μg of protein were subjected to Western blotting for iNOS. A: blot shown is representative of 6 Western blots. B: quantification of results from 6 Western blots. *P < 0.05 vs. IL-1 group.

Fig. 3. Role of an interferon-γ activation site (GAS) in rat iNOS promoter activation in RASMC. A: sequence of the GAS site EMSA probe (lead strand): −941 to −922 bp of rat iNOS promoter. The nucleotides of the GAS site (−936 to −928 bp) are underlined. B: EMSA were performed on nuclear extracts from RASMC after 30 min of treatment with vehicle, IL-1 (100 units/ml), IFN (250 units/ml), or both IL-1 and IFN. A specific antibody against STAT1α was used in the EMSA to identify the factor bound to the GAS probe. The gel shown is representative of 3 separate experiments. C: RASMC were stimulated with IL-1 (100 units/ml), IFN (250 units/ml), or both for 6 h following transient transfection of the wild type or GAS site-deleted (Del-GAS) −1.4-kb rat iNOS promoter-pGL3 constructs. Data are from 4 experiments (n = 12), each performed in triplicate, and are expressed as means ± SE. *P < 0.01 vs. wild type. D: the IFN-enhanced promoter activities were calculated by subtracting the IL-1-induced activity from the IL-1+IFN-induced promoter activity. Data are expressed as means ± SE (n = 12). *P < 0.01 vs. wild type.

All loss-of-function IRF site mutations abolished the IFN-γ-enhanced activity but not the IL-1β-induced activity (Fig. 5, A and B). A gain-of-function mutation of the IRF site also was generated. The IRF site was
changed to 100% homology with the IRF consensus sequence, and two additional such IRF sites were inserted upstream of the original IRF site (Ins2IRF). Ins2IRF increased the IFN-γ-enhanced activity but not the IL-1β-induced activity (Fig. 5, A and B). These results suggest that the IRF site mediates IFN-γ-enhanced iNOS expression and that the C/EBP site contributes to IL-1β-induced iNOS expression.

**Role of NF-κB activation in iNOS induction.** With the use of a commercial NF-κB probe (Fig. 6A) and nuclear extracts from RASMC, EMSA showed that IL-1β, but not IFN-γ, induced NF-κB binding to the probe. IFN-γ did not enhance the IL-1β-induced binding (Fig. 6B). Previously, we had demonstrated that a reverse NF-κB site mediates IL-1β-induced rat iNOS promoter activity in RASMC (37). As shown in Table 2, reverse NF-κB site mutations did not significantly affect the IFN-γ-enhanced activity. The results suggest that NF-κB activation mediates IL-1β-induced iNOS expression but not the IFN-γ-enhanced iNOS expression.

**DISCUSSION**

We have demonstrated that in RASMC, IL-1β induces iNOS expression, but IFN-γ only enhances it. We also have demonstrated that in RASMC, IL-1β, but not IFN-γ, induces NF-κB and C/EBP nuclear binding, whereas IFN-γ, but not IL-1β, induces STAT1 and IRF-1 nuclear binding. Although IFN-γ enhances iNOS expression induced by IL-1β, it does not enhance the IL-1β-induced nuclear bindings of NF-κB and C/EBP.

The JAK/STAT pathway is a well-characterized pathway of IFN-γ signaling (9). It involves IFN-γ dimer binding to IFN-γR1, IFN-γR1 and IFN-γR2 association, JAK1 and JAK2 activation, phosphorylation of Tyr457 in IFN-γR1, recruitment and phosphorylation of Tyr701 in STAT1α, STAT1α dimerization, and nuclear translocation. The final step of the JAK/STAT pathway is the interaction between the STAT1α dimer and a GAS element in the promoter regions of IFN-γ-inducible genes to initiate the induction of this family of genes. STAT1α activation and its binding to a GAS site in murine iNOS promoter have been shown to mediate iNOS induction by IFN-γ or LPS in RAW 264.7 cells (13); inhibition of JAK2 decreases iNOS mRNA induction in human DLD-1 cells (18). However, inhibition of the JAK/STAT pathway also enhances iNOS induction by IFN-γ plus LPS in RASMC (23), suggesting that the JAK/STAT pathway might play a different role in iNOS induction in RASMC. Rat and murine iNOS promoter have identical GAS sites. We have shown that IFN-γ, but not IL-1β, induces STAT1α binding to the GAS site in rat iNOS promoter. As in RAW 264.7
cells, the JAK/STAT pathway appears to mediate IFN-γ-enhanced iNOS promoter activation in RASMC, because deletion of the GAS site decreases the IFN-γ-enhanced promoter activity. The GAS element exhibits two functions in iNOS induction in RASMC. Besides mediating IFN-γ enhancement, it inhibits iNOS induction by IL-1β, because deletion of the GAS site increases IL-1β-induced promoter activity. The exact mechanism is not clear. It may be independent of the JAK/STAT pathway, because IL-1β itself does not induce STAT1α binding to the GAS site.

There are seven STATs (STAT1, 2, 3, 4, 5A, 5B, and 6) and four JAKs (JAK1, 2 and Tyk2). STAT1 has two splice variants; STAT1β, unlike STAT1α, lacks the terminal 38 amino acids and cannot restore IFN-γ responsiveness in U3 cells (8). The JAK/STAT pathway mediates not only IFN transduction but also that of a large variety of polypeptide ligands. STATs are phosphorylated not only by JAK kinases that are associated with cytokine receptors but also by receptors with intrinsic tyrosine activity (for example, epidermal growth factor, platelet-derived growth factor, and colony-stimulating factor-1 receptors). JAK kinases do not seem to have specificity for a particular STAT substrate and the specificity of receptor tyrosine kinase

Table 2. Reverse NF-κB mutations and the IFN-γ-enhanced promoter activity

<table>
<thead>
<tr>
<th></th>
<th>Wild Type</th>
<th>Mutant</th>
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<tr>
<td>Del-RNF-κB</td>
<td>109.3 ± 8.4 (n = 15)</td>
<td>126.6 ± 25.2 (n = 15)</td>
</tr>
<tr>
<td>RNF-κB-Mu3C</td>
<td>103.4 ± 11.5 (n = 9)</td>
<td>83.2 ± 7.0 (n = 9)</td>
</tr>
<tr>
<td>R-NF-κB-MuC</td>
<td>93.5 ± 10.1 (n = 12)</td>
<td>68.3 ± 11.3 (n = 12)</td>
</tr>
</tbody>
</table>

Data are expressed as means ± SE % of interleukin (IL)-1β-induced activity. Del-RNF-κB, reverse NF-κB site (−901 to −892 bp) deletion. RNF-κB-Mu3C, substitution of TTT for CCC at −894 to −892 bp. R-NF-κB-MuC, substitution of T for C at −894 bp. There are no significant differences between the mutated and the wild-type promoters.
activation of STATs is unclear (8). The complexity of the JAK/STAT pathway may explain why inhibition of the JAK/STAT pathway might superinduce iNOS induction by IFN-γ plus LPS in RASMC (23). The JAK/STAT pathway may not be an important mechanism for iNOS induction in RASMC because deletion of the GAS site does not significantly change the IFN-γ plus IL-1β-induced promoter activity (Fig. 3). The reasons behind the differences between the present study and that of Marrero et al. (23) are unclear. It is possible that the combination of LPS and IFN-γ used by Marrero et al. (23) may have a very different effect from the use of IL-1β and IFN-γ in the present study. There may be other signal transduction pathways mediating IFN-γ-enhanced iNOS expression in RASMC. For example, IFN-γ activates NF-κB in ChWK cells (38). Also, the GAS element appears to be dispensable for the expression of many IFN-γ-induced genes, whereas the IRF element is not (5).

IRF-1 has been shown to be important for iNOS induction (15). The IRF site mediates the IFN-γ-enhanced murine iNOS promoter induction in RAW 264.7 cells but has no effect on the LPS-induced murine iNOS promoter activity in RAW 264.7 cells (24). IRF-1 is induced by IFN-γ in RASMC (32). However, the relationship between IFN-γ-induced IRF-1 activation and iNOS induction in RASMC has not been established. In this study, IRF-1 is induced by IFN-γ but not by IL-1β. Similar to the murine iNOS promoter study (24), disruption of the IRF site abolishes only the IFN-γ-enhanced, but not the IL-1β-induced, rat iNOS promoter activity in RASMC. Similarly, insertion of two additional IRF sites increases the IFN-γ-enhanced NF-κB site does not significantly change IFN-γ-enhanced activity. We suggest that the C/EBP site contributes to IL-1β-induced iNOS expression in RASMC.

There are three NF-κB sites in the rat iNOS promoter, one upstream (−965 to −956 bp), one reverse (−901 to −892 bp), and one downstream (−107 to −98 bp). The up- and downstream NF-κB sites are required for iNOS induction in RASMC (41). IL-1β induces NF-κB. The reverse NF-κB site has been demonstrated to mediate the IL-1β-induced rat iNOS promoter activity in RASMC (37); however, disruption of the reverse NF-κB site does not significantly change IFN-γ-enhanced activity (Table 2). IFN-γ does not induce NF-κB nuclear binding and does not enhance IL-1β-induced NF-κB nuclear binding. It is unlikely that IFN-γ enhances iNOS expression in RASMC through the NF-κB pathway, although IFN-γ has been shown to activate NF-κB in ChWK cells (38).

There may be two ways to enhance IL-1β-induced iNOS transcription: enhancing the IL-1β-induced transcription factors that are responsible for iNOS gene transcription or inducing other transcription factors that cooperate with IL-1β-induced transcription factors to enhance the iNOS gene transcription. As shown in Fig. 7, our study demonstrates that in RASMC, IL-1β induces iNOS gene transcription through activation of NF-κB and C/EBP. IFN-γ does not induce or enhance the activation of NF-κB and C/EBP; however, it induces IRF-1 and STAT1 activation and may enhance the activation of IRF-1 and STAT1 through the JAK/STAT pathway. This may explain why inhibition of the JAK/STAT pathway abolished both IFN-γ-enhanced and IL-1β-induced rat iNOS promoter activity in RASMC; however, the IRF site mediates only the IFN-γ-enhanced activity. We suggest that the C/EBP site contributes to IL-1β-induced iNOS expression in RASMC.

Substitution of CC for AA at −910 to −909 bp of the murine iNOS promoter decreased IL-1 plus IFN-γ plus TNF-α-induced murine iNOS promoter activity in A7r5 cells, a rat vascular smooth muscle cell line (34). It is quite possible that the C/EBP site in rat iNOS promoter is functional. We have demonstrated that IL-1β, but not IFN-γ, induces C/EBP binding to the C/EBP site. The C/EBP site overlaps with an IRF site (−918 to −907 bp of the rat iNOS promoter). Disruption of both the IRF and C/EBP sites abolishes both IFN-γ-enhanced and IL-1β-induced rat iNOS promoter activity in RASMC; however, the IRF site mediates only the IFN-γ-enhanced activity. We suggest that the C/EBP site contributes to IL-1β-induced iNOS expression in RASMC.

Fig. 7. Schematic diagram of molecular mechanisms of iNOS promoter activation by IL-1β and IFN-γ in RASMC. Circled plus symbol represents stimulation.

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hance iNOS transcription through cooperation among IRF-1, STAT1, NF-κB, and C/EBP. In RASMC, like IL-1β, TNF-α strongly induces NF-κB; however, unlike IL-1β, TNF-α alone does not induce iNOS (41). This finding suggests that iNOS transcription in RASMC normally requires the cooperation of multiple transcription activation.

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