Src Activation of NF-κB Augments IL-1β–Induced Nitric Oxide Production in Mesangial Cells

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NF-κB is a critical transcription factor that is involved in glomerulonephritis and inflammatory host responses and a critical transactivator of the inducible nitric oxide (NO) synthase gene in mesangial cells. The Src protein tyrosine kinases (SFK) are involved in several signaling pathways and have been proposed to mediate cytokine activation of NF-κB in a few cell types. However, the specific involvement of SFK in IL-1β induction of NO production has not been clearly established. Accordingly, pharmacologic and molecular tools were used to clarify this issue in cultured murine mesangial cells. The SFK antagonist 4-amino-5-(4-chlorophenyl)-7-(t-butyl) pyrazolo(3,4-d)pyrimidine (PP2) dramatically inhibited IL-1β–mediated induction of endogenous NO production as measured by the Griess reaction, as well as the induction of NF-κB p50/p65 DNA-binding activity in gel shift assays and the activity of an NF-κB–responsive promoter–reporter construct transiently transfected into the cells. Immunoprecipitation and immunoblotting with anti-IκBα and anti-phosphotyrosine antibodies revealed that PP2 also inhibited IL-1β–stimulated tyrosine phosphorylation of IκBα, a requisite step in NF-κB activation in this signaling cascade. In agreement with the pharmacologic inhibition studies, siRNA directed against c-Src specifically limited c-Src protein expression and inhibited IL-1β–mediated induction of NF-κB DNA-binding activity, whereas control siRNA had no effect. Conversely, overexpression of constitutively active c-Src augmented basal and IL-1β–mediated induction of NF-κB DNA-binding activity and NO production. Thus, SFK play a key role in IL-1β–induced NO production in mesangial cells and do so via tyrosine phosphorylation of IκBα and consequent NF-κB activation.


Nitric oxide (NO) is an important molecular mediator of numerous physiologic processes in virtually every organ. In the kidney, NO plays prominent roles in the homeostatic regulation of glomerular, vascular, and tubular function. During glomerular inflammation, NO and cytokines such as IL-1β and TNF-α from resident and infiltrating cells act in concert to sustain, promote, or limit glomerular damage. NO that is produced by these cells leads to activation or silencing of genes that encode antioxidant defense enzymes, matrix-metabolizing enzymes, proinflammatory mediators, and signaling mechanisms (1). In human glomerulonephritis, inducible nitric oxide synthase (iNOS) gene expression has been described in glomerular mesangial cells, as well as in local and infiltrating macrophages (2,3). Mesangial cells contribute prominently to the pathogenesis of glomerulonephritis, in part by producing a variety of cytokines and NO via iNOS, and there is evidence for participation of iNOS-generated NO in the induction, progression, or protection of several types of experimental and human glomerulonephritis. Proinflammatory stimuli, such as IL-1β (4), activate iNOS gene transcription in mesangial cells through a complex network of signaling pathways and inducible transcription factors, including c-Jun N-terminal kinase (JNK) and p38 mitogen-activated protein kinase (5), cAMP response element binding protein (CREB) (4), CCAAT-enhancer binding protein (C/EBPβ) (4), and NF-κB (6). Of these, NF-κB is considered the most potent transactivator of the iNOS gene and has been the subject of more intense investigation.

NF-κB is a pivotal transcription factor that regulates genes that control multiple immune and inflammatory responses, as well as cell proliferation and apoptosis (7). NF-κB is activated in several forms of experimental and human glomerulonephritis, including immune complex kidney disease (8–11), crescentic glomerulonephritis (12), and lupus nephritis (9), and it activates iNOS in these settings. A variety of inflammatory stimuli, such as IL-1β (13,14), bacterial lipopolysaccharide and IFN-γ (14), and oxidative stress (15) activate this transcription factor in mesangial cells. NF-κB consists of a group of five proteins, namely c-Rel, RelA (p65), RelB, NF-κB1 (p50 and p105), and NF-κB2 (7). In the resting state, NF-κB is sequestered in the cytoplasm through a tight association with specific inhibitory proteins, called inhibitors of NF-κB (IκB). The most comprehensively studied pathway of NF-κB activation by agents such as cytokines, endotoxin, and phorbol esters consists of IκBα phosphorylation at Ser-32 or Ser-36 by the IκBα kinase (IKK), which targets IκBα for ubiquitination and degradation through the ubiquitin-26S proteasomal pathway (16,17), allowing nu-
clear translocation of NF-κB, where the transcription factor binds to specific DNA sequences located in the promoter regions of many proinflammatory genes. The majority of stimuli that lead to NF-κB activation act through this classical pathway (16,18,19).

Two other pathways have been described to activate NF-κB in distinct cell types. The first pathway has been described in lymphocytes that were subjected to oxidant stress (20,21) or pervanadate treatment (22) and involves phosphorylation of Tyr-42 but not Ser-32 or Ser-36 of I kBα. The dissociation of tyrosine-phosphorylated I kBα from NF-κB in this setting is not the result of degredation by the 26S proteasome (23). This pathway seems to be specific for I kBα, because Tyr-42 is not conserved in other IκB family members. The exact protein tyrosine kinase(s) or phosphatase(s) involved in this pathway remains unknown, but members of the Src family of nonreceptor tyrosine kinases (SFK), such as p56Lck, ZAP-70, and SFK (24–27), have been implicated. Moreover, Tyr-42 phosphorylation of I kBα occurs within a consensus binding site for the p85 subunit of phosphatidylinositol 3-kinase and seems to stabilize its affinity for p85 (28). SFK have also been implicated in the regulation of cytokine-mediated NF-κB activation in T helper 2 cells (25) and bone marrow macrophages (24). The second atypical pathway occurs in cells that are exposed to short-wavelength ultraviolet radiation and involves I kBα degradation by the proteasomal pathway without phosphorylation of Ser-32, Ser-36, or Tyr-42 of I kBα (29,30). The mechanisms for this alternative pathway of I kBα degradation are unknown.

IL-1β signaling in mesangial cells activates multiple pathways that are involved in iNOS expression—p38 mitogen-activated protein kinase (31), JNK/stress-activated protein kinase (SAPK) (31), and phosphatidylinositol 3-kinase (32)—and these can be inhibited by pharmacologic blockade in an NF-κB–independent manner (31). We have also shown that NF-κB plays a complex role in transcriptional regulation of iNOS in murine mesangial cells (13,14). In this report, we sought to determine additional signaling events in the NF-κB pathway that are critical to iNOS activation in these cells. We demonstrate that IL-1β–mediated induction of iNOS-mediated NO production in murine mesangial cells proceeds through SFK tyrosine phosphorylation of I kBα and consequent activation of NF-κB.

Materials and Methods

Cell Culture and Reagents

Mouse mesangial cells (American Type Culture Collection CRL-1927, Manassas, VA) were maintained in Ham’s F-12 plus DMEM supplemented with 2 mM L-glutamine, and 5% FBS. Mouse recombinant IL-1β was obtained from R&D Systems (Minneapolis, MN). The SFK inhibitor 4-amino-5-(4-chlorophenyl)-7-((t-butyl) pyrazolo(3,4-d)pyrimidine (PP2 [33]) was obtained from Calbiochem (San Diego, CA). Polyclonal antibodies that recognize NF-κB p65 and p50, as well I kBα, and an NF-κB consensus oligomer (5’-AGTTCAGGGGACTTTCCAGGC-3’; consensus sequence boldface) were obtained from Santa Cruz Biotechnologies (Santa Cruz, CA). Lipofectamine 2000 reagent was purchased from Invitrogen (Carlsbad, CA). The Dual Luciferase Reporter Assay system and the luciferase vectors pGL3-Basic and pRL-SV40 were from Promega (Madison, WI). The protein estimation kit was purchased from Bio-Rad (Hercules, CA). The Silencer siRNA Transfection Kit, siPORT Amine Transfection Reagent, and predesigned siRNA targeting exon 5 of murine SFK protein tyrosine kinase (ID #160390) were purchased from Ambion (Austin, TX). Monoclonal anti–c-Src antibody was obtained from Dharmaco (Chicago, IL).

Plasmids

The NF-κB promoter–reporter construct p36B(–) (NF-κB3-luc, which contains three tandem copies of the NF-κB–binding element (GGG-GACTCTCCCCCCC) upstream of the SV40 early promoter sequence and fused to the coding sequence for the luciferase gene, was provided by Dr. Bharat Aggarwal (The University of Texas M.D. Anderson Cancer Center, Houston, TX). We have used this construct in our previous publication (14). Constitutively active chicken SFK (34) subcloned into pCMV5 (35), designated here pCMV5–c-Src, was provided by Dr. Jeffrey Frost (University of Texas Health Science Center at Houston, Houston, TX).

Nitrite Assays

Mesangial Cells were seeded in 24-well plates and transiently transfected in some experiments or treated with inhibitors in other experiments as indicated in the text or figure legends, and then treated with IL-1β at a dose of 10 ng/ml for 24 h. The medium then was collected, and the nitrite concentration was determined with the Griess Reagent system (Promega) according to the manufacturer’s protocol.

Transient Transfections

Mesangial Cells were seeded in 24-well plates and grown to 90 to 95% confluence in complete medium without antibiotics and transfected the next day using Lipofectamine 2000 reagent according to the manufacturer’s protocol and a total of 1 μg/well of plasmid DNA. The amount of DNA was kept constant by adding appropriate amounts of the parental empty expression vector. Transfection efficiencies were normalized by co-transfection with 10 ng/well of the Renilla luciferase expression plasmid pRL-SV40. Twenty-four hours after transfection, complete medium was added with vehicle or IL-1β. Twenty-four hours later, cell lysates were prepared and firefly and Renilla luciferase activities were measured in 100 μl of lysate samples as described previously by our laboratory (36). In some experiments, as indicated in the text and figure legends, PP2 (10 μM/ml) was added after transfection for 24 h before the cell lysates were prepared. This concentration of PP2 was chosen on the basis of its effectiveness in inhibiting tyrosine kinase activities in previously published studies (26,37).

Preparation of Nuclear Extracts and Electrophoretic Mobility Shift Assays

Nuclear extracts were prepared from time-paired vehicle and IL-1β–treated mesangial cells as detailed in our previous work (36,38). NF-κB element double-stranded oligonucleotides were end-labeled with γ-32P-ATP (3000 Ci/mmol) using T4 polynucleotide kinase. Binding reactions were performed in 20 μl of solution for 30 min at room temperature by incubating 10 μg of nuclear extract protein with duplex DNA probe (2 × 105 cpm in reaction buffer [13 mM HEPES (pH 7.9), 65 mM NaCl, 0.14 mM EDTA, 1 mM MgCl2, 1 mM dithiothreitol, 8% glycerol, and 50 μg/ml poly[dI-dC]]) for supershift assays, antibodies (2 μg) that are specific for NF-κB p65 or p50 were added to the binding reaction and incubated on ice for 10 min before the addition of the labeled probe. Aliquots of the reaction were resolved in 5% native polyacrylamide gels in 0.5× Tris-borate EDTA buffer. The gels were dried and exposed to x-ray film with an enhancing screen at ~70°C to detect the DNA–protein and DNA–protein–antibody complexes. Ex-
periments were replicated a minimum of three times as indicated in the figure legends.

**Immunoprecipitation and Immunoblot Analysis of Total and Tyrosine-Phosphorylated IκBα**

The procedures followed were generally described in our previous work (39). In brief, stimulated mesangial cells were harvested and lysed in lysis buffer (20 mM Tris-HCl [pH 8.0], 1.5 mM MgCl₂, 0.2 mM EDTA, 25% Glycerol, and 0.5 mM PMSF). These lysates were then precleared with 20 μl/ml protein A/G agarose beads (Santa Cruz) for 1 h at 4°C. After brief centrifugation, the supernatant was added to anti-IκBα antibody (0.2 μg/ml) lysis buffer overnight at 4°C, followed by the addition of protein A/G agarose beads and incubation for 1 h at 4°C. Immunoprecipitates were washed four times in lysis buffer and resuspended in SDS sample buffer, boiled for 5 min, and analyzed on 4 to 20% SDS-PAGE gels. The proteins were electrophoretically transferred to polyvinylidene difluoride membranes (Hybond-ECL; Amersham, Piscataway, NJ) and probed with anti-IκBα for identification of total IκBα levels or with anti-phosphotyrosine antibodies (0.2 μg/ml) for identification of tyrosine phosphorylated IκBα overnight as indicated in the text and figure legends. The blots were washed extensively with TBST solution that contained 50 mM Tris (pH 8.0), 138 mM NaCl, 2.7 mM KCl, and 0.05% Tween 20. The antigen–antibody complexes were detected by the ECL protocol using horseradish peroxidase–conjugated donkey anti-rabbit IgG as secondary antibody.

c-Src Silencing by RNA Interference

Cultured murine mesangial cells were grown in complete medium until 90% confluent. Before transfection, the medium was changed to serum- and antibiotic-free medium (Opti-MEM). The c-Src–specific or control siRNA was combined with siPORT Amine Transfection Reagent, and the cells were transfected according to the recommended protocol with siRNA (50 nM final concentration). Six hours after transfection, the medium was changed, and 48 h were allowed before IL-1β treatment. Successful, specific gene knockdown was determined by immunoblot analysis of c-Src and glyceraldehyde-3-phosphate dehydrogenase protein levels.

**Statistical Analyses**

Quantitative data are presented as mean ± SEM and were analyzed either by paired t test or ANOVA as appropriate. Significance was assigned at P < 0.05.

**Results**

**SFK Antagonist PP2 Inhibits IL-1β–Mediated Induction of Endogenous NO Production and iNOS Expression**

We showed previously that iNOS is the only NOS isoform expressed in these cells and that IL-1β-stimulated nitrite production correlates exclusively with iNOS gene expression, and iNOS-generated NO (14,36,38). Accordingly, we used IL-1β–stimulated NO production as a convenient index of iNOS gene regulation. To determine whether SFK activity contributes to the regulation of iNOS-generated NO production in mesangial cells, we measured nitrite production induced by treatment with IL-1β (10 ng/ml) in the presence of vehicle or PP2, a potent and specific SFK inhibitor (40). As shown in Figure 1, IL-1β–stimulated mesangial cells that had been treated with 10 μM/ml PP2 exhibited nitrite levels that were approximately half that of IL-1β–stimulated controls. From the same cells used for the nitrite determinations, we also analyzed iNOS protein expression. As seen in Figure 2A, no iNOS was apparent in the absence of IL-1β regardless of treatment with vehicle or PP2. iNOS was sharply induced by IL-1β, and this was substantially blunted by co-administration of PP2, in agreement with the nitrite results.

**PP2 Inhibits IL-1β–Mediated Induction of NF-κB DNA-Binding Activity and the Activity of an NF-κB–Responsive Promoter**

NF-κB is known to play a major role in cytokine induction of the murine iNOS promoter. To determine whether SFK participate in this signaling pathway, we tested the effects of PP2 treatment on NF-κB DNA-binding activity and the activity of an NF-κB–responsive promoter–reporter construct in mesangial cells. Gel shift studies demonstrated two sequence-specific κB DNA–protein complexes in nuclear extracts under basal conditions (Figure 2A). Both complexes were upregulated after IL-1β treatment, but this response was markedly greater for complex I. Moreover, this complex was supershifted by antibodies to either the p50 or the p65 subunits of NF-κB (Figure 2B), indicating that the complex contained p50/p65 heterodimers. PP2 treatment of the cells significantly reduced the intensity of complex I, reflecting reduced NF-κB DNA-binding activity, in the nuclear extracts that were prepared from IL-1β–stimulated mesangial cells (Figure 2A). In keeping with this observation, PP2-treated cells exhibited approximately 40% lower rates of IL-1β–stimulated activity of the NF-κB promoter–reporter compared with vehicle-treated controls (Figure 2C). Collectively, these results suggest that SFK activity is critical for the DNA-binding properties and transactivation potential of NF-κB and iNOS induction in IL-1β–stimulated mesangial cells.

**PP2 Inhibits IL-1β–Stimulated Tyrosine Phosphorylation of IκBα**

To determine whether tyrosine phosphorylation of IκBα is important for IL-1β activation of NF-κB, we measured protein...
levels of total IκBα and phosphotyrosine-IκBα in mesangial cell lysates from control, IL-1β + vehicle-treated, PP2-treated, and IL-1β + PP2-treated mesangial cells. Low levels of total IκBα were present in the control and PP2-treated cells, whereas comparably high levels were present in the IL-1β-treated cells in the presence or absence of PP2. Phosphotyrosine-IκBα, detected by immunoprecipitation of total IκBα followed by immunoblotting with anti-phosphotyrosine antibody, was evident in control cells, strongly induced in IL-1β-treated cells, at control levels in the PP2-treated cells, and less than control levels in the IL-1β + PP2-treated cells (Figure 3). These results were consistent with the variations in NF-κB DNA-binding activity (Figure 2A).

siRNA Knockdown of c-Src Protein Expression in Mesangial Cells Inhibits IL-1β-Mediated Induction of NF-κB DNA-binding Activity

Mesangial cells were transfected with either siRNA targeting c-Src or an equimolar amount of negative control siRNA and then treated with IL-1β. Western blots of cytosolic extracts showed that c-Src levels in cells that were treated with IL-1β alone or with IL-1β + negative control siRNA were similar. However, cells that were transfected with c-Src siRNA revealed substantially less c-Src protein (Figure 4A). In contrast, glyceraldehyde-3-phosphate dehydrogenase protein levels, used as a specificity control, were invariant across the samples (Figure 4A). Electrophoretic mobility shift assay that was performed on nuclear extracts revealed no effects of the negative control siRNA on IL-1β–induced NF-κB DNA-binding activity but diminished IL-1β induction of NF-κB DNA-binding activity in c-Src siRNA transfected cells compared with controls (Figure 4B), in keeping with the pharmacologic inhibition studies with PP2 (Figure 2A).

c-Src Overexpression Augments Basal and IL-1β-Mediated Induction of NF-κB DNA-Binding Activity and NO Production

Immunoblots of cytosolic extracts from cells that were transfected with pCMV5-c-Src encoding constitutively active c-Src revealed the expected overexpression of c-Src compared with the vector-transfected cells regardless of treatment with vehicle or IL-1β (Figure 5A). Gel shift assays demonstrated increased NF-κB DNA-binding activity both in the basal state and in response to IL-1β in the cells that overexpressed constitutively active c-Src as compared with empty vector-transfected controls (Figure 5A). Similarly, overexpression of constitutively active IκBα protein expression in the presence or absence of PP2. Phosphotyrosine-IκBα, detected by immunoprecipitation of total IκBα followed by immunoblotting with anti-phosphotyrosine antibody, was evident in control cells, strongly induced in IL-1β-treated cells, at control levels in the PP2-treated cells, and less than control levels in the IL-1β + PP2-treated cells (Figure 3). These results were consistent with the variations in NF-κB DNA-binding activity (Figure 2A).

Figure 2. PP2 inhibits IL-1β induction of NF-κB DNA-binding activity and transactivation potential. (A) Cytosolic and nuclear extracts were prepared from mesangial cells that had been treated with vehicle, IL-1β, PP2, or IL-1β + PP2. Cytosolic extracts were immunoblotted (IB), with intervening membrane stripping, with antibodies directed against inducible NO synthase (iNOS) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Nuclear extracts were subjected to electrophoretic mobility shift assay (EMSA) with a κB-specific consensus oligomer. Sequence-specific DNA–NF-κB complexes I and II are indicated. Blots are representative of three observations. (B) Supershift assay in which antibodies directed against NF-κB p50 or p65, or nonimmune IgG were included in the binding reactions performed as in A. The supershift bands “SS” are designated. (C) Mesangial cells were transiently co-transfected with p36B(−)[(NF-κB)3-luc, which contains three tandem copies of the κB binding element, together with the Renilla luciferase expression plasmid pRL-SV40. The cells were treated with IL-1β and vehicle or PP2 for 24 h. The luciferase activity then was measured in cell lysates. *P < 0.05 versus IL-1β alone; n = 5.

Figure 3. PP2 inhibits tyrosine phosphorylation of IκBα. Aliquots of whole-cell lysate protein were immunoprecipitated (intraperitoneally) with anti-IκBα Ab, followed by immunoblotting (IB) with an anti-phosphotyrosine Ab (anti–Tyr-P) to detect tyrosine phosphorylation on IκBα. The blots were stripped and reprobed with an anti-IκBα antibody for estimation of total IκBα levels. A representative (n = 3) blot is shown.
active c-Src by transient transfection resulted in higher expression of iNOS protein (Figure 5A) and rates of nitrite generation (Figure 5B) under both basal and IL-1β/H9252–treated conditions compared with controls. The increment was greater in the IL-1β–treated cells.

Discussion

Resting mesangial cells produce low basal levels of inflammatory mediators, including NO, but soluble factors that are produced by inflammatory cells that invade the glomerulus or by circulating factors can activate iNOS and stimulate NO production. The signaling mechanisms that are activated by cytokines and lead to induction of iNOS gene transcription are complex and incompletely defined. In this report, we explored SFK as an upstream component that leads to activation of NF-κB, a transcription factor that is known to be a strong transactivator of the iNOS gene in multiple cell types, including mesangial cells. Through pharmacologic and molecular manipulation of SFK activity and expression, we found that SFK significantly activate tyrosine phosphorylation of IκB, NF-κB DNA-binding activity and transactivation potential, iNOS protein expression, and iNOS-generated NO production in mesangial cells. Cells that were treated with the SFK inhibitor PP2 exhibited notable blunting of iNOS gene expression and NO production, NF-κB DNA-binding activity, and NF-κB–dependent promoter activity in response to IL-1β. In agreement with these results, siRNA knockdown of c-Src resulted in similar blunting of IL-1β induction of NF-κB DNA-binding activity. Conversely, overexpression of constitutively active c-Src in mesangial cells augmented basal and IL-1β–mediated induction of iNOS, NO generation, and NF-κB–binding activity.

These actions correlated with SFK-mediated tyrosine phosphorylation of IκBα. Collectively, these results more clearly define a new pathway for iNOS induction, involving cytokine activation of SFK, consequent IκBα tyrosine phosphorylation, and NF-κB activation, that ultimately results in induction of iNOS-generated NO production.

To date, studies that have evaluated the functional regulation of IκBα tyrosine phosphorylation and NF-κB regulation have been restricted to a few cell types, including T cells (22,25), bone marrow–derived macrophages (24), and epithelial cells (21,37). Abu-Amer et al. (24) demonstrated in bone marrow–derived macrophages that SFK tyrosine phosphorylation was essential in TNF-α–mediated activation of NF-κB through a mechanism that involved tyrosine phosphorylation of IκBα but did not involve IκBα degradation. Similarly, Natarajan et al. (41) showed that NF-κB activation in human endothelial cells that were subjected to hypoxia/reperfusion injury required tyrosine

Figure 4. siRNA knockdown of c-Src inhibits IL-1β–stimulated NF-κB DNA-binding activity. Mesangial cells were transfected with control siRNA or c-Src–specific siRNA and then exposed to vehicle or IL-1β for 24 h. (A) Immunoblots of cytosolic extracts were prepared and probed with an anti-c-Src antibody, then stripped and reprobed with an anti-GAPDH antibody. (B) Nuclear extracts were assayed for NF-κB DNA binding activity by EMSA. Blots are representative of three observations.

Figure 5. Overexpression of c-Src augments basal and IL-1β–stimulated NF-κB DNA-binding activity and NO production. Twenty-four hours after transient transfection with constitutively active c-Src expression plasmid or empty vector, mesangial cells were treated with vehicle or IL-1β. Twenty-four hours later, cytosolic extracts were immunoblotted (IB), with intervening membrane stripping, with antibodies directed against iNOS, c-Src, and GAPDH, and nuclear extracts were assayed for NF-κB DNA binding activity by EMSA (A), and the medium supernatant was assayed for nitrite concentration (B). *P < 0.05 versus control vector + vehicle; #P < 0.05 versus control vector + IL-1β; n = 4.
phosphorylation of IkBa, which in turn did not induce IkBa proteolysis. In Th2 cells, the SFK member Lck was shown to be important for IL-1β-induced NF-κB activation (25) by a mechanism that did not involve IkBa degradation (25). Huang et al. (37), however, examined the involvement of these proteins in TNF-α-induced cyclooxygenase-2 expression and found that SFK-mediated activation of NF-κB was associated with rapid degradation of IkBa. Hence, the exact mechanism through which SFK participates in relaying cytokine-induced cell signaling that leads to NF-κB activation seems to be cell type and stimulus specific.

The role of NF-κB in iNOS transcriptional control is complex. The iNOS 5′-flanking region contains at least two κB enhancer elements that bind NF-κB and mediate strong transactivation of the iNOS gene (42). In addition, we previously demonstrated that NF-κB p65 physically and functionally interacts with histone deacetylase-2 (14) and STAT3 (13) in mesangial cells to augment and inhibit, respectively, NF-κB-mediated transactivation of the iNOS gene and endogenous NO production in cultured glomerular mesangial cells. In the case of STAT3, the mechanism(s) did not impair the ability of NF-κB to bind its cognate elements in the iNOS promoter and did not require STAT3 itself to bind directly to the promoter. These transcription factor interactions seem to be nuclear events downstream of SFK and IkBa actions, although it remains plausible that SFK-mediated tyrosine phosphorylation of other target proteins, including STAT3 itself (43), may govern these protein–protein interactions and the ultimate degree of NF-κB-mediated induction of the iNOS gene. For example, if both IkBa (shown here) and STAT3 (shown in other settings [43]) were targets of SFK-mediated tyrosine phosphorylation, then the degree of NF-κB activity might depend on whether SFK-mediated tyrosine phosphorylation of STAT3 impaired or accentuated its binding interaction with NF-κB p65. Further studies will test this hypothesis. Furthermore, that PP2 was effective in blocking NF-κB activation indicates that the tyrosine kinase activity of SFK was important for the effect. These results differ from those of Funokoshi-Tago et al. (26), who found that SFK tyrosine kinase activity was not required for IL-1β-induced NF-κB activation in a glioblastoma cell line, apparently because of a physical and functional interaction between SFK and IkBa kinase-γ.

Because IL-1β triggered tyrosine phosphorylation of IkBa, it is reasonable to conclude that IL-1β acts through both serine and tyrosine phosphorylation of IkBa to regulate NF-κB-mediated cell signaling. Given that c-Src overexpression in mesangial cells also significantly increased basal NO production and NF-κB DNA-binding activity, it seems that c-Src-mediated tyrosine phosphorylation alone is necessary and sufficient to activate NF-κB and consequently the iNOS gene in these cells. Further studies will be needed to elucidate the exact mechanism involved in this process. Similar findings were observed in a human glioblastoma cell line in which overexpression of SFK induced a moderate increase in NF-κB activity in the absence of cytokine stimulation (26). This is not unique to c-Src, as overexpression of other signaling components in the IL-1β-dependent pathway, such as TRAF6, have been reported to induce NF-κB activation in the absence of cytokine stimulation (44).

In the resting state, NF-κB typically is bound to various IkBa proteins. Only IkBa in this family of proteins has a tyrosine residue at position 42 (28,41). This association between NF-κB and IkBa may allow NF-κB to respond specifically to certain stimuli that induce tyrosine phosphorylation, allowing cell type- and stimulus-specific cell signal modulation. Likewise, iNOS transcription has cell- and stimulus-specific controls. This diversity of regulatory mechanisms provides considerable versatility in activating, sustaining, and inactivating potent inducers such as NF-κB and iNOS. That the inhibition of NO production and NF-κB activation by PP2 and c-Src knockdown and the basal and IL-1β-stimulated activation of NO production by overexpressed c-Src were so prominent indicates that the data reported here add another key component to the regulatory schemes for these proteins.

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