Regulation of Cytokine-inducible Nitric Oxide Synthase in Cardiac Myocytes and Microvascular Endothelial Cells

ROLE OF EXTRACELLULAR SIGNAL-REGULATED KINASES 1 AND 2 (ERK1/ERK2) AND STAT1α*

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Adult rat ventricular myocytes and cardiac microvascular endothelial cells (CMEC) both express an inducible nitric oxide synthase (iNOS or NOS2) following exposure to soluble inflammatory mediators. However, NOS2 gene expression is regulated differently in response to specific cytokines in each cell type. Interleukin-1β (IL-1β) induces NOS2 in both, whereas interferon γ (IFN-γ) induces NOS2 expression in myocytes but not in CMEC. Therefore, we examined the specific signal transduction pathways that could regulate NOS2 mRNA levels, including activation of 44- and 42-kDa mitogen-activated protein kinases (MAPKs; ERK1/ERK2) and STAT1α, a transcriptional regulatory protein linked to cell membrane receptors. Although IL-1β treatment increased ERK1/ERK2 activities in both cell types, IFN-γ activated these MAPKs only in myocytes. STAT1α phosphorylation, consistent with IFN-γ-induced signaling, was readily apparent in both cell types, and binding of activated STAT1α from cytoplasmic or nuclear fractions from IFN-γ-treated adult myocytes to a sis-inducible element could be demonstrated by gel-shift assay. The farnesylintransferase inhibitor BZA-SB blocked activation of ERK1/ERK2 and induction of NOS2 by IFN-γ and IL-1β in myocytes. IL-1β and IFN-γ-induced NOS2 gene expression in myocytes was also down-regulated by both protein kinase C (PKC) desensitization and by the PKC inhibitor bisindolylmaleimide, implicating PKC-linked activation of Ras or Raf in the induction of NOS2 by IL-1β and IFN-γ in cardiac muscle cells. In CMEC, the MAPK kinase inhibitor PD 98059 blocked activation of ERK1/ERK2 and down-regulated IL-1β-mediated NOS2 induction, whereas activation of ERK2 in the absence of cytokines by okadaic acid, an inhibitor of phosphoserine protein phosphatases, also induced NOS2 mRNA.

These data demonstrate that ERK1/ERK2 activation appears to be necessary for the induction of NOS2 by IL-1β and IFN-γ in cardiac myocytes and CMEC. In the absence of ERK1/ERK2 activation by IFN-γ in CMEC, phosphorylation of STAT1α is not sufficient for NOS2 gene expression. These overlapping yet distinct cellular responses to specific cytokines may serve to target NOS2 gene expression to specific cells or regions within the heart and also provide for rapid escalation of NO production if required for host defense.

Both cardiac myocytes and microvascular endothelial cells isolated from adult rat ventricular muscle express the cytokine-inducible form of nitric oxide synthase (iNOS or NOS2) both in vivo and in primary culture, although the regulation of NOS2 gene expression in response to specific cytokines is regulated differently in these two cell types. Interleukin-1β (IL-1β) treatment induces NOS2 in both cell types, whereas interferon γ (IFN-γ) induces NOS2 in ventricular myocytes but not in CMEC. However, IFN-γ does augment NOS2 induction by IL-1β in CMEC (1–5). To gain insight into the mechanisms regulating NOS2 induction by cytokines in both cell types, we studied two distinct signal transduction pathways: activation of p44/p42 mitogen-activated protein kinases (MAPKs; extracellular signal-regulated kinases, ERK1/ERK2) (6–10), and the tyrosine phosphorylation of STAT1α (signal transducer and activator of transcription-1; STAT1α) (11). The murine macrophage NOS2 gene promoter region has been shown to contain two AP-1 sites for which trans-acting transcriptional factors are regulated by MAPKs and three IFN-γ-activated sites for STAT1α binding (12).

The 44- and 42-kDa MAPK (ERK1/ERK2) isoforms are ubiquitously expressed serine/threonine protein kinases, activated by dual specificity MAPK kinases (MEK1/MEK2) in response to diverse stimuli. A number of receptor tyrosine kinases, cytokine receptors, and heterotrimeric G proteins have been shown to activate MEK1/MEK2 and MAPKs (10, 13, 14). In neonatal rat cardiac myocytes, several endogenous hypertrophic stimuli have also been shown to activate MAPKs (15–20). Among other actions, activated MAPKs translocate to the nucleus (21, 22), where they can phosphorylate downstream kinases that directly activate transcription factors.

A number of cytokines (e.g. IFNs, IL-6, leukemia inhibitory factor, and colony stimulating factor 1) and growth factors (e.g. epidermal growth factor and platelet-derived growth factor) have been shown to tyrosine phosphorylate STAT1α through the activation of Janus family kinases, Jak1 and Jak2 (11, 23–25). The STAT family of signal transduction proteins are substrates for the JAK kinases, with specific STAT isoforms...
acting to provide specificity for cytokine receptor-mediated signaling. Depending on the identity of the activated cytokine receptor and JAK recruited to the membrane, specific STAT isoforms form either heterodimers or homodimers and bind to promoter elements of specific genes. With IFN-γ signaling, activated STAT1α forms homodimers, translocates to the nucleus, and binds to IFN-γ-activated site elements of IFN-γ-responsive genes (26, 27).

In this report, we present evidence that activation of ERK1/ERK2 (MAPKs) is essential for the induction of NOS2 gene expression in response to IL-1β and IFN-γ in adult rat ventricular myocytes and cardiac microvascular endothelial cells. Activation of STAT1α itself is not sufficient for NOS2 gene expression, although it can act synergistically to increase NOS2 mRNA in the presence of activated ERK1/ERK2 in both cell types.

**EXPERIMENTAL PROCEDURES**

Cell Isolation and Culture—Ventricular myocytes were isolated from hearts of adult male Sprague-Dawley rats (175–200 g) as described previously (28). Briefly, hearts were perfused retrogradely with normally Ca²⁺-free Krebs Henseleit bicarbonate (KHB) buffer and were minced and dissociated with this KHB buffer containing trypsin (0.02 mg/ml) and deoxyribonuclease (0.02 mg/ml). The cell mixture was filtered and sedimented twice through a 6% bovine serum albumin cushion to remove nonmyocyte cells. The cell pellet was suspended and plated in (DMEM; Life Technologies, Inc.) supplemented with albumin (2 mg/ml), L-carnitine (2 mM), creatine (5 mM), taurine (5 mM), and 0.1% penicillin streptomycin (defined medium) on laminin (1 μg/ml)-coated dishes. For assay of MAPK activity, myocytes were washed twice and grown in defined medium for 24 h before treatment with cytokines. For northern analyses, myocytes were treated with cytokines 4 h after plating and harvested after 16 h of treatment.

CMEC from adult rat hearts were isolated as described by Nishida et al. (29). Briefly, after removing the outer ventricular wall, the remaining tissue was finely minced and treated with collagenase and trypsin in Ca²⁺-free Hank’s balanced salt solution (Life Technologies, Inc.). Dissociated cells were washed and resuspended in DMEM containing 20% fetal calf serum and antibiotics and plated on laminin (1 μg/ml)-coated dishes. After reaching confluency, CMEC were serum-starved for 24 h before treatment with reagents for MAPK and STAT1α assays. For northern analyses, confluent cells were serum-starved for 4 h before harvesting with cytokines or okadaic acid for 16 h.

In-Gel MAPK Assay—To assess the activation of MAPKs, in-gel mobility shift assay (MBP) kinase assays were carried out as described by Wang and Erickson (30). Cell extracts were prepared by lysis with buffer A (1% Triton X-100, 150 mM NaCl, 10 mM Tris pH 7.4, 1 mM EDTA, 1 mM EGTA, 0.2 mM sodium vanadate, 0.2 mM phenylmethylsulfonyl fluoride, and 0.5% Nonidet P-40), and total protein content was measured by Bradford assay (Bio-Rad). Protein samples (100 μg) were resolved by SDS-PAGE using a 7.5% gel. The gel was fixed and proteins were transferred to nitrocellulose membrane (Schleicher & Schuell). The tyrosine-phosphorylated proteins were detected by rabbit polyclonal anti-phosphotyrosine antibodies. The immunoprecipitates were separated on 7.5% SDS-PAGE and autoradiography.

Tyrosine Phosphorylation of STAT1α—The total protein (500 μg) from either ventricular myocytes or CMEC cell lysates was immunoprecipitated with anti-STAT1α (p91) antibodies (Transduction Laboratories). The immunoprecipitates were separated on 7.5% SDS-PAGE gels (Bio-Rad), and the proteins were transferred to a nitrocellulose membrane (Schleicher & Schuell). The tyrosine-phosphorylated proteins were detected by rabbit polyclonal anti-phosphotyrosine antibodies (Transduction Laboratories) as the primary antibody and an anti-rabbit antibody linked to horseradish peroxidase (Pierce) as the secondary antibody. Rabbit antibodies were then detected by chemiluminescence reagents (DuPont NEN).

Electrophoretic Mobility Shift Assay—Cytoplastic and nuclear fractions from ventricular myocytes were prepared as described by Thieren and Drouin (32). Briefly, hearts were perfused with 10 mM Tris, pH 7.5; 50 mM NaCl, 1 mM dithiothreitol, 1 mM EDTA, 5 mM MgCl₂, 4 μg poly(dI-dC), and 5% glycerol containing 500,000 cpm of the double-stranded oligonucleotide probe with the sequence 5′-GATCAGCTTCATTTCCCGTAAATCCCTA-3′ (35) filled in by a Klenow fragment of DNA polymerase I with [α-32P]dCTP. This oligonucleotide has the consensus sequence for IFN-γ-activated sites (i.e. TTTCNNNAA (11)) and has shown to bind both STAT1α and a sis-inducible factor. Underlined sequences indicate a region of dyad symmetry shared by IFN-γ-activated sites and sis-inducible element (6). The reaction was allowed to proceed at room temperature for 30 min or for an additional 10 min when anti-STAT1α antibodies were added. The reactions were electrophoresed on a 4% polyacrylamide gel and analyzed by autoradiography.

RNA Isolation and Northern Analysis—Total RNA from cells was extracted by the method of Chomczynski and Sacchi (33). RNA was size-fractionated on 1.0% formaldehyde agarose gels and transferred to gene screen plus membranes (DuPont NEN) using a vacuum blotter (Bio-Rad 785). The blots were then hybridized overnight to a radilabeled probe at 42°C. A NOS2 cDNA (1) or c-fos cDNA were labeled using a random prime DNA labeling kit (Boehringer Mannheim). The blots were washed twice for 5 min each with 2 × SSC (1 × SSC = 0.1 M NaCl and 0.01 M sodium citrate) at room temperature twice for 30 min each with 2 × SSC containing 0.1% SDS at 60°C, and twice again for 15 min each with 0.05 × SSC at room temperature prior to autoradiography. Normalization of RNA for equal loading was carried out by hybridizing the blots with radiolabeled oligonucleotide complementary to 18 S rRNA (34) or with a geralddehyde-phosphate dehydrogenase cDNA probe. Radioactivity of the autoradiograph was quantitated by densitometric analysis using a Pharmacia Biotech 2202 Uitroscan laser densitometer.

RESULTS

Activation of ERK1/ERK2 in Cardiac Myocytes by IL-1β and IFN-γ—In order to verify that adult rat ventricular myocytes, which cannot re-enter the cell cycle, also have the ability to activate ERK1/ERK2 MAPKs, phorbol 12-myristate 13-acetate, a tumor promoting agent (TPA) was used. Primary isolates of adult ventricular myocytes that had been cultured for 24 h in defined medium were exposed to 200 ng/ml TPA over a range of times from 5 to 120 min. Cell lysates were prepared and analyzed by in-gel MAPK assay using MBP as a substrate. The results in Fig. 1 show the activation of 44- and 42-kDa MAPKs within 5 min of exposure to TPA. The activation of MAPKs starts to diminish after 60 min of treatment with TPA. The solvent dimethyl sulfoxide alone does not activate MAPK over control (0 min). The positions of both the 44- and 42-kDa MAPK (ERK1/ERK2) isoforms are recognized by a radilabeled geralddehyde-phosphate dehydrogenase cDNA probe. To exclude a nonspecific toxic effect of specific reagents on cell viability, representative blots were rehybridized to a 32P-labeled geralddehyde-phosphate dehydrogenase cDNA probe. Radioactivity of the autoradiograph was quantitated by densitometric analysis using a Pharmacia Biotech 2202 Uitroscan laser densitometer.

Earlier reports from this laboratory have documented that cytokines (e.g. IFN-γ, IL-1α, and IFN-γ + IL-1β) can induce the expression of NOS2 in adult rat ventricular myocytes (1). We examined the ability of these cytokines to activate MAPK in these cells, as shown in Fig. 2A. Untreated cardiac myocytes cultured in serum-free defined medium have some detectable
ERK1/ERK2 activity is increased in IFNγing of activated STAT1 proteins (12), we examined STAT1 and CMEC shown to contain three IFNκines in CMEC. A 91-kDa protein. Neither phenylephrine nor IL-1βinduction with 4 ng/ml rhIL-1β, or the α-adrenergic agonist phenylephrine (10 μM) with 1 μM nonselective (Phes). Total cell lysates (100 μg) were analyzed by in-gel MBP kinase assay, and the positions of the p42/p44 MAPKs on the gel are shown.

ERK1/ERK2 activity at baseline using the in-gel MAPK assay technique, and this activity could be enhanced by a 15-min exposure of these cells to either cytokine and to the α-adrenergic agonist phenylephrine as a positive control. Both rmIFNγ (500 units/ml) and rhIL-1β (4 ng/ml) activated MAPKs over control cells. To verify these results obtained by the in-gel MAPK assay, the activation of ERK1/ERK2 by IFNγ was further studied by an immune complex MAPK assay. Cell lysates prepared after a 15-min exposure to IFNγ were immunoprecipitated by anti-ERK2 antibodies. Immune complexes were then incubated with a reaction mixture containing MBP and [γ-32P]ATP. The results shown in Fig. 2B confirm that ERK1/ERK2 activity is increased in IFNγ-treated cells when compared with untreated cells. As expected, an increase in c-fos mRNA levels could be detected within 30 min of addition of IFNγ to myocyte primary isolates (Fig. 2C).

Activation of ERK1/ERK2 in CMEC in Response to Cytokines—This laboratory has shown that CMEC can express NOS2 when treated with IL-1β alone but not when treated with IFNγ alone (3, 4). To study the activation of ERK1/ERK2 in these cells, confluent CMEC primary cultures that had been serum-starved for 24 h were treated either with IFNγ or IL-1β or with a combination of IFNγ and IL-1β for 15 min. IL-1β and the combination of IL-1β + IFNγ activated ERK1/ERK2 significantly (Fig. 3A). However, 500 units/ml of rmIFNγ treatment did not activate these MAPKs significantly over control (Fig. 3A). Because the maximum activation of ERK1/ERK2 was noted at 15 min with either IL-1β alone or the combination of IL-1β and IFNγ in a time course experiment (data not shown), it appears that IFNγ alone fails to activate MAPKs or induce NOS2 expression in CMEC. IFNγ alone also failed to increase c-fos mRNA to detectable levels in CMEC within 30 min of exposure to the cytokine (Fig. 3B).

Activation of STAT1α (p91) by Cytokines in Cardiac Myocytes and CMEC—Because the NOS2 gene promoter region has been shown to contain three IFNγ-activated site elements for binding of activated STAT1 proteins (12), we examined STAT1α activity in both ventricular myocytes and CMEC primary cultures after 15 min of exposure to IL-1β and/or IFNγ. Fig. 4 (A and B) show that rmIFNγ (500 units/ml) alone or in combination with 4 ng/ml rhIL-1β induces tyrosine phosphorylation of a 91-kDa protein. Neither phenylephrine nor IL-1β alone promoted STAT1α phosphorylation in myocytes under the conditions employed here. Similarly, in CMEC IFNγ but not IL-1β activates STAT1α phosphorylation (Fig. 4C). Because IFNγ did not activate ERK1/ERK2 or induce NOS2 expression in CMEC, activation of STAT1α does not seem to be sufficient for inducing NOS2 gene expression in these cells.

To determine whether this phosphorylated STAT isoform can bind DNA, electrophoretic mobility gel-shift assays were carried out (Fig. 5). A protein from the nuclear as well as cytoplasmic fractions prepared from IFNγ-treated adult ventricular myocytes bound the radiolabeled oligonucleotide designed to bind STAT1α, whereas no protein from control cytoplasmic or nuclear fractions bound this oligonucleotide. The identity of factor(s) responsible for altering the mobility of the oligonucleotide was confirmed by supershift of this protein by anti-STAT1α (p91) antibodies. Thus, in cardiac myocytes IFNγ induces activation of ERK1/ERK2 and activation and binding of STAT1α to DNA elements.

Inhibition of ERK1/ERK2 Prevents NOS2 Induction by Cy-
BZA-5B inhibited both IL-1 downstream signaling. A 15-min pretreatment of cells with other proteins, thereby inhibiting normal Ras function and attachment of a farnesyl moiety to some Ras isoforms, among activated MAPKs with the induction of NOS2 gene expression, we additional 10 min.

Effect of the farnesyl transferase inhibitor BZA-5B on ERK1/ERK2 activation and NOS2 mRNA levels in cytokine-treated ventricular myocytes. A, BZA-5B inhibits activation of ERK1/ERK2 by IFNγ. Ventricular myocytes were exposed to defined medium containing rmIFNγ (500 units/ml, IFN) or defined medium containing 25 μM BZA-5B (BZA) for 15 min. Cells were pretreated for 15 min with 25 μM of BZA-5B and then with rmIFNγ (500 units/ml) for another 15 min (IFN, BZA). Total cell lysates were then analyzed by the in-gel MBP kinase assay. The positions of p42/p44 MAPKs were identified by using CMED exposed to DMEM containing 10% fetal calf serum for 10 min (Serum). The experiment was performed twice with similar results. B, BZA-5B inhibits NOS2 induction by IFNγ. Ventricular myocytes were exposed to defined medium alone (lane C) or defined medium containing 500 units/ml of IFNγ (IFN) for 16 h. Cells were pretreated with 25 μM BZA-5B for 1 h and then exposed to rmIFNγ (500 units/ml) for 16 h (IFN, BZA). Total cellular RNA was then used for Northern blot analysis using NOS2 cDNA and 18S rRNA probes. This experiment was performed twice with similar results. C, BZA-5B inhibits activation of ERK1/ERK2 by IL-β. Ventricular myocytes were exposed to defined medium alone (lane C) or defined medium containing 15 μM Okadaic acid (Okadaic acid) or defined medium containing 4 ng/ml of IL-β (IL-β) for 16 h. Cells were pretreated with 25 μM BZA-5B for 1 h and then exposed to IL-β (4 ng/ml) for 16 h (IL-β, BZA). Total cellular RNA was then used for Northern blot analysis using NOS2 cDNA and 18S rRNA probes.

BZA-5B in combination with IFNγ almost completely inhibited the increase in NOS2 gene expression above control levels, whereas IL-β-induced NOS2 gene expression was reduced by approximately 60% (Fig. 6, B and D). BZA-5B at this concentration for 16 h had no obvious effect on myocyte viability (i.e. cells remained rod-shaped with clear cross-striations and attached to plates), nor did it affect the expression of glyceraldehydephosphate dehydrogenase by Northern blot (data not shown). In contrast to these results in cardiac myocytes, BZA-5B had no consistent effect on the extent of ERK1/ERK2 activation in CMEC in response to cytokines and appeared to enhance rather than inhibit the extent of NOS2 induction in these cells (data not shown).

Activation of ERK1/ERK2 and Induction of NOS2 Expression in CMEC—To further investigate the association of ERK1/ERK2 activation with NOS2 induction in CMEC, confluent serum-starved endothelial cells were treated with okadaic acid alone or in combination with IFNγ. Okadaic acid, a relatively specific inhibitor of phosphatidic protein phosphatases 1, 2a, 4, and 5 that does not activate a PKC, has been shown to activate MAPKs in rat embryonic fibroblasts and a rat phagocytoma cells (PC12 cells) (37, 38). Fig. 7A shows that treatment of CMECs with okadaic acid alone activates ERK2 in a time-dependent manner, with no additional activation noted with IFNγ. To determine if okadaic acid-induced activation of MAPK could also induce NOS2 gene expression, Northern analyses were performed on CMEC pretreated for 16 h with okadaic acid. Fig. 7B shows that treatment of CMEC cells with
At concentrations above 50 ng/ml of TPA for 24 h before adding 4 ng/ml of IL-1β (IL-1) for a further 16 h. Cells were pretreated with 50 ng/ml TPA for 24 h and then exposed to 4 ng/ml of IL-1β (TPA, IL-1) in the same medium for 16 h. The cells in TPA lane were exposed to 50 ng/ml TPA for 40 h. B. Effect of down-regulation of PKC by TPA on IL-1γ-induced NOS2 expression. Ventricular myocytes were exposed to defined medium alone (lane C) or left untreated for 24 h before adding 4 ng/ml of IL-1α (IL-1) for a further 16 h. Cells were pretreated with 50 ng/ml TPA for 24 h and then exposed to 4 ng/ml of IL-1β (TPA, IL-1) in the same medium for 16 h. The cells in TPA lane were exposed to 50 ng/ml TPA for 40 h. C. Effect of BIM on cytokine-induced NOS2. Ventricular myocytes were pretreated with 500 nm of BIM for 1 h before treating cells with 4 ng/ml of IL-1α (BIM, IL-1) or with 500 units/ml of IFNγ (BIM, IFNγ) for 16 h. The cells were also treated with 4 ng/ml of IL-1α alone (IL-1) or 500 units/ml of IFNγ alone (IFNγ) for 16 h. Lane C represents untreated cells. In all cases, total cellular RNA was used for Northern blot analysis using NOS2 cDNA and 18 S rRNA probes.

To examine further the role of PKCs in NOS2 induction, both the nonselective protein kinase inhibitor H7 and bisindolylmaleimide (BIM), a relatively selective inhibitor of Ca2+-dependent PKC isoenzymes (42, 43), were used. H7 prevented induction of NOS2 in cardiac myocytes by IFNγ (data not shown). Treatment of myocytes with 500 nm BIM in combination with IFNγ inhibited NOS2 induction by approximately 50% (Fig. 8C). However, BIM had no effect on NOS2 induction by IL-1β in cardiac myocytes (Fig. 8C) or in CMEC (data not shown).

DISCUSSION

The data reported here suggest that in response to IL-1β or IFNγ, induction of NOS2 expression in cardiac myocytes and microvascular endothelial cells, two of the most prevalent cell types in heart muscle, requires activation of 44- and 42-kDa MAP kinases (ERK1/ERK2). This conclusion is based on the following observations: 1) IL-1β and IFNγ independently activate ERK1/ERK2 and increase NOS2 mRNA abundance in cardiac myocytes; 2) IL-1β but not IFNγ activates ERK1/ERK2 and increases NOS2 mRNA levels in CMEC; 3) inhibition of IFNγ- and IL-1β-linked signaling proteins leading to activation of ERK1/ERK2 in cardiac myocytes (i.e. PKCs and Ras) also inhibited IFNγ- and IL-1β-induced NOS2 expression in these cells; 4) nonreceptor-mediated activation of ERK2, induced by the phosphosine protein phosphatase inhibitor okadaic acid, induced NOS2 expression in CMEC; and 5) inhibition of IL-1β-induced activation of MEK and ERK1/ERK2 in CMEC by PD 98059 also suppressed NOS2 induction in these cells.

The role of ERK1/ERK2 in NOS2 induction was somewhat unexpected, due to recent reports that growth promoting factors known to activate MAPKs in a number of different cell types, such as angiotensin II, basic fibroblast growth factor, and phorbol esters, decrease NOS2 mRNA levels (44–48). Although the decline in cytokine-induced NOS activity with
growth factors could be correlated with entry into the cell cycle and increased cellular proliferation in some reports, this did not appear to be the explanation in one report of confluent serum-starved rat aortic smooth muscle cells exposed to inflammatory cytokines (47). However, in PC12 cells nerve growth factor, which is known to induce ERK1/ERK2 in these cells, has been reported recently to increase transcription of several NOS isoforms, including NOS2, suggesting that one or more NOS isoforms could be acting as a growth arrest gene, initiating the switch to cytostasis during differentiation (49–51). Also, in inflammatory cells (murine peritoneal macrophages), induction of NOS2 by lipopolysaccharide correlated with ERK2 (p42 MAPK) phosphorylation. Both effects of lipopolysaccharide could be inhibited by the tyrphostin class of tyrosine kinase inhibitors (52).

Activation of ERK1/ERK2 MAPKs alone cannot be sufficient for NOS2 induction by cytokines. Phorbol esters, which activate diacylglycerol-responsive PKC isoforms and which subsequently can induce Ras/Raf-mediated activation of MEK1/ERK2 and PKC-dependent and PKC-independent mechanisms in different cell types (53, 54). In cardiac myocytes, IL-1β-induced NOS2 expression is in part dependent on PKC activation, whereas in CMEC, IL-1β induction of NOS2 appears to be mediated by PKC-independent mechanisms. IL-1β, which does not activate STAT1α but does increase ERK1/ERK2 activities in both cell types, is also known to activate NF-κB signaling in many different cell types (52, 55). This pathway is likely to play a role in NOS2 induction by IL-1β in both cardiac myocytes and in CMEC.

In adult cardiac myocytes (i.e., cells that are not competent to re-enter the cell cycle), IFNγ activates both ERK1/ERK2 and STAT1α signaling pathways. The nuclear factor from IFNγ-treated myocytes that bound to a double-stranded oligonucleotide was positively identified by a STAT1α antibody-induced supershift on gel-shift assay (Fig. 5). This is presumably mediated by recruitment to type II cytokine receptors of JAK1 and/or JAK2 phosphotyrosine kinases that subsequently tyrosine phosphorylate and activate STAT1α (27). Angiotensin II also has been shown recently to activate STAT1α/β (p59/p84) following JAK2 phosphorylation in rat aortic smooth muscle cells and in neonatal rat cardiac fibroblasts, although the time course of JAK2 tyrosine phosphorylation in smooth muscle cells was significantly shorter than with activation of this pathway by IFNγ (56, 57). Both epidermal growth factor and platelet-derived growth factor, cytokines that initiate intracellular signal transduction at phosphotyrosine kinase receptors, have been reported to activate STAT1α in Swiss 3T3 cells, and epidermal growth factor activates STAT3 in rat aortic smooth muscle cells (56). However, epidermal growth factor did not activate STAT1α signaling in adult cardiac myocytes and microvascular endothelial cells using the experimental conditions we describe here.²

² K. Singh, R. A. Kelly, and T. W. Smith, unpublished data.

The pathway(s) by which IFNγ activates ERK1/ERK2 in cardiac myocytes is not known. The ability of the farnesyl transferase inhibitor BZA-58 to inhibit ERK1/ERK2 activation by IFNγ suggests Ras-mediated membrane recruitment and activation of Raf-1 (MAPK kinase kinase or MEK kinase). All four Ras proteins and Raf-1 are farnesylated at CAAX motifs (ras is also myristoylated), although there appear to be important differences among the Ras proteins in their sensitivity to this drug (58, 59). Also, inactivation of Raf has not been demonstrated to date with BZA-58. However, the ability of phorbol ester pretreatment (i.e., PKC desensitization) and of bisindolylmaleimide to block IFNγ-mediated induction of NOS2 suggests that activation of a diacylglycerol-regulated PKC isoform is required (60). Type II cytokine receptors (i.e., type II interferon receptors α/β) are not trimeric G protein-coupled receptors that could initiate phospholipid signaling by activating phospholipase Cβ isoforms. Nor are they phosphotyrosine kinases that could recruit proteins with src-homology (SH2) domains, such as phospholipase Cγ isoforms or phosphoinositide 3-kinase (61, 62). However, after receptor activation and oligomerization, phosphotyrosine kinases such as JAK could phosphorylate tyrosine residues on the cytosolic domain of these receptors, which could then initiate phospholipid signaling and PKC activation after binding and activation of phospholipase Cγ and other proteins (27). Although not proven by the data reported here, it is likely that activation of phospholipid signaling and STAT1α recruitment and phosphorylation together act to induce NOS2 expression with IFNγ in ventricular myocytes.

In microvascular endothelial cells, IL-1β-mediated activation of ERK1/ERK2 could be blocked by the MEK inhibitor PD 98059 and the extent of NOS2 induction reduced by 70%, suggesting that CMEC IL-1β-induced NOS2 gene expression is at least partially dependent on Ras/Raf-mediated signaling. Although IFNγ alone does not induce ERK1/ERK2 phosphorylation or increase NOS2 mRNA abundance in CMEC, we have shown previously that this cytokine accelerates the time course and extent of NOS2 mRNA accumulation and protein activity in combination with IL-1β (3, 4). The ability of IFNγ to also potentiate the increase in NOS2 mRNA accumulation induced by the phosphoserine protein phosphatase inhibitor okadaic acid, which acts directly to inhibit dephosphorylation of MEKs and ERK1/ERK2 downstream from PKC, supports the notion that IFNγ signaling is mediated by a non-ERK1/ERK2-dependent signaling pathway in these cells, one of which is presumably mediated by STAT1α. The suppression of IFNγ-induced NOS2 expression in cardiac myocytes also exposed to okadaic acid emphasizes the differences among cell types in the balance of activities of protein kinases and phosphatases (63).

The apparent redundancy of IL-1β and IFNγ signaling in ventricular myocytes, at least with respect to NOS2 expression coupled with important differences in signaling initiated by these cytokines in other cell types such as CMEC, termed pleiotropy by Taniguchi (27), is likely necessary to provide specificity and to regulate the intensity of host defense mechanisms. If the phenotype of these endothelial cells is low passage, confluent primary cultures that are representative of the capillary endothelium in vivo, which does express NOS2 abundantly in several experimental animal models (3, 64), then cell type-specific cytokine signaling for NOS2 induction probably occurs in situ within cardiac muscle as well. This would make sense biologically because selectively increased microvascular endothelial production of NO or related congeners would elicit both local vasodilation and increased vascular permeability, among other actions that are necessary for the early stages of an inflammatory response. Unrestricted production of NO by infiltrating inflammatory cells and/or by microvascular endothelial cells can directly impair the contractile function of adjacent cardiac myocytes, as has been shown in short term primary heterotypic culture models (4). In addition, high levels of NOS2 induction in the heart, which appear to occur with high blood and tissue levels of cytokines, as occurs in the systemic inflammatory response syndrome, and which may occur in cardiac allograft rejection as well, will result in global
dysfunction of the heart and is often clearly detrimental to the organism (65). Therefore, the selective activation of ERK1/ERK2 we observed in response to IFN-γ in cardiac myocytes in vitro but not in microvascular endothelial cells illustrates one mechanism by which the expression could be limited to a specific cell type within the heart and other tissues.

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