Role of Interferon Regulatory Factor-1 and Mitogen-activated Protein Kinase Pathways in the Induction of Nitric Oxide Synthase-2 in Retinal Pigmented Epithelial Cells

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Bovine retinal pigmented epithelial cells express an inducible nitric oxide synthase (NOS-2) after activation with interferon-γ (IFN-γ) and lipopolysaccharide (LPS). Experiments were performed to investigate the involvement of interferon regulatory factor-1 (IRF-1) on NOS-2 induction and its regulation by NO inhibitors such as pyrrolidine dithiocarbamate (PDTC), an antioxidant, or protein kinase inhibitors. Analysis by transitory transfections showed that LPS, alone or with IFN-γ, stimulated activity of the murine NOS-2 promoter fragment linked upstream of luciferase and its suppression by PDTC and by the different protein kinase inhibitors, genistein (tyrosine kinase inhibitor), PD98059 (mitogen-activated protein (MAP) kinase kinase inhibitor), and SB 203580 (p38 MAP inhibitor). Using specific antibodies, we have confirmed that extracellular signal-regulated kinases and p38 MAP kinase were activated by LPS and IFN-γ in retinal pigmented epithelial cells. Analysis by reverse transcriptase-polymerase chain reaction, Western blot, and electrophoretic mobility shift assay demonstrated that IFN-γ alone or combined with LPS induced an accumulation of IRF-1 mRNA and protein and IRF-1 DNA binding. Transfections assays with the IRF-1 promoter showed an induction of this promoter with IFN-γ, potentiated by LPS. The decrease of LPS/IFN-γ-induced IRF-1 promoter activity, IRF-1 synthesis, and IRF-1 activation, by PDTC, genistein, PD98059, and SB 203580, could explained in part the inhibition of the NOS-2 induction by these compounds. Our results demonstrate that IRF-1 is necessary for NOS-2 induction by LPS and IFN-γ and that its synthesis requires the involvement of a redox-sensitive step, the activation of tyrosine kinases, and extracellular signal-regulated kinases 1/2 and p38 MAP kinases.

The enzyme nitric oxide synthase (NOS) transforms l-arginine into nitric oxide (NO) and l-citrulline in the presence of oxygen, NADPH, tetrahydrobiopterin, flavin mononucleotide, and FAD (1, 2). Three isoforms of NOS have been identified. Two isoforms are expressed continuously: NOS-1 is present essentially in neurons of the central and peripheral nervous system (3), and NOS-3 is localized originally in the plasma membrane of vascular endothelial cells (4). These enzymes, via an increase of the intracellular calcium concentration, produce small amounts of NO, which are involved in neurotransmission and vasorelaxation (3, 4). On the other hand, the inducible isoform, NOS-2, whose expression requires protein synthesis, is calcium- and calmodulin-independent and is generally expressed in different cell types only after transcriptional activation by endotoxins or cytokines (5, 6). NO produced by NOS-2 plays a role in immunological defenses as an antitumoral, antimicrobial, and antiviral agent (5–7). NO is also considered to be a mediator of autoimmune and inflammatory responses (5).

In the retina, Müller glial cells can express NOS-2 after endotoxin and cytokine stimulation (8). Retinal pigmented epithelial (RPE) cells from bovine (9), human (10), and murine (11, 12) species also contain the NOS-2 isoform. Indeed, we demonstrated previously that in bovine RPE cells, NOS-2 was induced by combined treatment with lipopolysaccharide (LPS) and interferon-γ (IFN-γ) but not individually (9). The regulation of NOS-2 induction is dependent on signal transduction activated by endotoxin, cytokines, and growth factors (6, 13). These signals are the result of the activation of serine/threonine or tyrosine kinases (14, 15). In bovine RPE cells, the accumulation of NOS-2 mRNA and the accompanying NO release induced by LPS and IFN-γ require tyrosine kinase signaling and oxidative mechanisms (16). We have also demonstrated that the transcription factor nuclear factor-κB (NF-κB) is required in LPS/IFN-γ-induced NOS-2 mRNA accumulation in bovine RPE cells (16) as in many different cell types such as murine macrophages (17, 18), vascular smooth muscle cells (19), or 3T3 fibroblasts (20). In RPE cells, the antioxidant pyrrolidine dithiocarbamate (PDTC) but not the tyrosine kinase inhibitor genistein reduces the nuclear translocation of NF-κB and the formation of NF-κB-DNA complexes induced by LPS/IFN-γ (16). Furthermore, the fact that LPS/IFN-γ-induced NOS-2 mRNA accumulation is sensitive to cycloheximide (16) suggests that transcriptional factors that depend on protein synthesis are required for NOS-2 induction in RPE cells. In this context, different studies have reported the necessity of interferon regulatory factor-1 (IRF-1), transcriptionally induced by IFN-γ, in NOS-2 gene induction (21, 22). In bovine RPE cells, we have demonstrated that IRF-1 mRNA accumulation can be modulated after IFN-α and IFN-γ treatment (23), also suggesting a role for this transcription factor in NOS-2 induction in RPE cells. In this study we have attempted to elucidate further the role of IRF-1 in NOS-2 induction in bovine RPE cells. Our results demonstrate that NOS-2 inducers (LPS/IFN-γ) increase
IRF-1 mRNA and protein accumulation and induce the formation of IRF-1-DNA complexes. Analysis of the effects of NOS-2 inhibitors, genistein, PDTC, and inhibitors of extracellular signal-regulated kinase (ERK) and p38 mitogen-activated protein (MAP) kinase pathways reveal that these compounds block LPS/IFN-γ-induced IRF-1 mRNA accumulation and suppress the LPS/IFN-γ-stimulated activity of IRF-1.

MATERIALS AND METHODS

Cell Cultures—Bovine RPE cells were prepared as reported previously (16). They were cultured in DMEM supplemented with 10% fetal calf serum (Life Technologies, Cergy-Pontoise, France), 2.5 μg/ml fungizone, 50 μg/ml gentamycin, and 2 mM l-glutamine. Cultures were homogeneous and contained only RPE cells, as characterized by immunohistochemistry with anti-cytokeratin monoclonal antibody KL-1 (24). Cells at passages 1–5 were used for experiments. Retinal Müller glial (RMG) cells were cultured from eyeballs from mice at postnatal day 10 according to Hicks and Courtouy (25). Early subcultures (up to three passages) were used for transfection experiments.

Chemicals, Cytokines, and Antibodies—LPS from Salmonella typhimurium, PDTC, and genistein were obtained from Sigma (St. Quentin-Fallavier, France). The specific inhibitor of MAP kinase kinase (PD98059), the upstream kinase that phosphorylates and activates ERK kinases, and a specific inhibitor of p38 MAP kinase (SB 203580), were first tested on NO production. After different times of incubation, total RNA was extracted from treated cells by cell lysis in guanidinium isothiocyanate followed by phenol acid extraction. 1 μg of RNA was reverse transcribed for 90 min at 42 °C with 200 units of superscript Moloney murine leukemia virus reverse transcriptase (Life Technologies), using random hexamers. 2 μl of cDNA was added to each PCR, and amplification was performed with the oligonucleotide primers specific for mouse IRF-1, and GAPDH as described previously (23, 29).

Statistical Analysis—Results were expressed as mean ± S.E. They were analyzed statistically by Mann-Whitney U test. p values less than 0.05 were considered as significant.

RESULTS

Transfection of Bovine RPE Cells with the NOS-2 Promoter—Because we have demonstrated previously that the accumulation of NOS-2 mRNA by the combination of LPS and IFN-γ could be impaired by antioxidants and tyrosine kinase inhibitors (16), we attempted to establish whether these effects were the result of the prevention of NOS-2 gene transcription. For this, we decided to analyze the induction of the promoter of NOS-2 by transitory transfection of the NOS-2 promoter linked to the luciferase gene reporter. Transfected cells were stimulated for 12 h to measure the maximal luciferase activity (Fig. 1A). When bovine RPE cells were stimulated with LPS (bar 2), it appeared that there was an increase of the luciferase activity; none was observed in unstimulated cells (bar 1) or in cells stimulated with IFN-γ alone (bar 3). When cells were stimulated with the combination of LPS and IFN-γ (bar 4), the luciferase activity was similar to that observed with LPS alone. Control experiments revealed that no luciferase activity was detected in LPS/IFN-γ-stimulated RPE cells transfected with the promoterless pGL2-basic construct, and a large increase of luciferase activity in unstimulated cells transfected with the SV40 early promoter/enhancer pGL2-control (data not shown).

Western Blot Analysis—For MAP kinase analysis, RPE cells were serum starved for 48 h before stimulation. After a 2-h pretreatment with PDTC or protein kinase inhibitors, cells were treated with LPS and IFN-γ for distinct periods, washed with phosphate-buffered saline, and then scraped into lysis buffer as described (23). Samples were counted, and 100 μg of protein per lane of each sample was subjected to SDS-polyacrylamide gel electrophoresis. Proteins were then transferred to an Immobilon membrane (Millipore, St. Quentin en Yvelines, France) by electroblotting. Western blot analysis using different polyclonal antibodies specific for IRF-1, total p38 MAP kinase, active p38 MAP kinase, and active ERK kinases was performed as described previously (23). The specific inhibitor of p38 MAP kinase (SB 203580), was added to each lane of a representative experiment (bar 2) and then scraped into lysis buffer as described previously (23). The Western blot analyses were performed as described previously (23, 29). A greater increase of the luciferase activity by LPS/IFN-γ stimulation rather than by LPS alone was observed with AP1 (bar 4 compared with bar 2), suggesting a difference in the induction of the promoter reporter in bovine RPE cells and murine RMC cells.

Inhibitory Effects of PD98059 and SB 203580 on Nitrile Accumulation and NOS-2-regulated Luciferase Activity Induced by LPS/IFN-γ in RPE Cells—To investigate the type of protein kinase pathway which could be involved in NOS-2 induction in RPE cells, a specific inhibitor of MAP kinase (PD98059), the upstream kinase that phosphorylates and activates ERK kinases, and a specific inhibitor of the p38 MAP kinase (SB 203580), were first tested on NO production.
Role of IRF-1 in NOS-2 Induction

Table I
Effect of genistein, PD98059, and SB 203580 on nitrite accumulation and NOS-2 promoter induction by LPS/IFN-γ

| Treatment                        | % of stimulation | % of maximal |
|---------------------------------|------------------|--------------|
| Control                         | 2 ± 1            | 100          |
| LPS + IFN-γ                     | 100              | 100          |
| LPS + IFN-γ + genistein         | 11 ± 3**         | 12 ± 4**     |
| LPS + IFN-γ + PD98059           | 44 ± 5**         | 35 ± 7**     |
| LPS + IFN-γ + SB 203580         | 35 ± 4**         | 13 ± 3**     |

For nitrite determination, measured by Griess reaction as described under “Materials and Methods,” cells were incubated with or without 1 μg/ml LPS and 100 units/ml IFN-γ in combination with 10 μM genistein, 10 μM PD98059, or 25 μM SB 203580 for 72 h. For the luciferase activity, after transitory transfections RPE cells were pretreated for 2 h without or with 90 μM genistein, 10 μM PD98059, or 25 μM SB 203580 and then stimulated with 1 μg/ml LPS and 100 units/ml IFN-γ in combination with genistein, PD98059, or SB 203580 for 12 h. Values (means ± S.E.) are expressed as a percentage of maximal nitrite accumulation or maximal luciferase activity after LPS and IFN-γ treatment. ** indicates p < 0.01, very significantly different from LPS/IFN-γ.

FIG. 1. Induction of NOS-2 promoter and regulation by genistein and PDTC. Panel A, bovine RPE cells, after transitory transfections, were pretreated without (bars 1–4) or with PDTC (bars 5 and 7) or genistein (bars 6 and 8) and then stimulated for 12 h without (bar 1) or with 1 μg/ml LPS (bar 2), 100 units/ml IFN-γ (bar 3), LPS and IFN-γ (bar 4), LPS with 10 μM PDTC (bar 5), LPS with 90 μM genistein (bar 6), LPS/IFN-γ with PDTC (bar 7), or LPS/IFN-γ with genistein (bar 8). Panel B, mouse RME cells, after transitory transfections, were stimulated for 12 h without (bar 1) or with 1 μg/ml LPS (bar 2), 100 units/ml IFN-γ (bar 3), or LPS/IFN-γ (bar 4). In each case, the luciferase activity was measured as described under “Materials and Methods.” The results are expressed as a multiplicative factor compared with the luciferase activity detected in nontreated RPE cells. The means ± S.E. of three experiments run in triplicate are shown. **p < 0.01 versus nontreated cells (bar 1) and **p < 0.01 versus LPS/IFN-γ treated cells (bar 4).

FIG. 2. Effect of genistein and PDTC on the IRF-1 activation. Cells were incubated for 12 h with different combinations of stimulants: medium alone (lane 1), 1 μg/ml LPS (lane 2), 100 units/ml IFN-γ (lane 3), LPS/IFN-γ and 90 μM genistein (lane 4), LPS/IFN-γ (lanes 5 and 7), and LPS/IFN-γ and 10 μM PDTC (lane 6). When used, PDTC and genistein were added to the cells 2 h before stimulation. Cell extracts were prepared and analyzed for IRF-1 binding activity in the EMSAs. Excess of unlabeled oligonucleotide was added to verify the specificity of complex formation (lane 7). The experiment shown represents one of three independent EMSAs that gave similar results.

caused by LPS and IFN-γ. As shown in Table I, the stimulated NO release determined from the nitrite level in the culture supernatants was decreased by 56 and 65% in the presence of PD98059 or SB 203580, respectively. Transfection experiments demonstrated that stimulation of the transfected cells with LPS/IFN-γ after pretreatment with PD98059 reduced luciferase activity by 65% (Table I). Similar treatment with SB 203580 also prevented LPS/IFN-γ-induced luciferase activity by 87% (Table I). These results suggest that the signaling pathway involved in NOS-2 induction is affected by inhibitors of ERK and p38 MAP kinase pathways.

Effects of Genistein and PDTC on IRF-1 Activation—Because we have recently described the activation of IRF-1 by inducers of NOS-2 (23), we tested the effect of genistein and PDTC on LPS/IFN-γ-induced IRF-1 activation. EMSA studies (Fig. 2) revealed the presence of one major and two minor DNA-protein complexes in extracts of RPE cells stimulated with IFN-γ alone (lane 3) or by the coaddition of LPS and IFN-γ (lane 5). These complexes were absent in control (lane 1) and in LPS-treated cells (lane 2). The formation of these complexes was prevented by the addition of excess unlabeled IRF-1 oligonucleotide (lane 7), demonstrating the specificity of the DNA-protein interaction. The middle darker complex appears to correspond to the probe complexed with IRF-1, whereas the fainter bands might represent other members of the IRF family such as IRF-2 (30). The amount of the complexes observed after the LPS/IFN-γ stimulation decreased in the presence of genistein (lane 4) or PDTC (lane 6), indicating that tyrosine kinase inhibitor and antioxidant induced a decrease of IRF-1 binding to its specific DNA target sequence.
maximal signal was observed after 3 or 6 h of stimulation (Fig. 4B). Analysis of the effects of the different inhibitors of NOS-2 induction revealed that IRF-1 mRNA accumulation induced by LPS/IFN-γ after 3 h of stimulation was decreased by pretreatment with PDTC or genistein (Fig. 5A). More specific analysis with the two distinct MAP kinase inhibitors (Fig. 5B) demonstrated that pretreatment with PD98059 (lane 3) or SB 203580 (lane 5) at a concentration that inhibited NOS-2 expression prevented the LPS/IFN-γ-induced IRF-1 mRNA accumulation.

**Induction and Regulation of the Promoter of IRF-1**—To address whether the inhibitory effects of the antioxidants and protein kinase inhibitors on IRF-1 mRNA accumulation were caused by the prevention of IRF-1 gene transcription, we decided to analyze the induction of the promoter of IRF-1 (Fig. 6) by transitory transfection assays with two different constructs of murine reporter gene of the IRF-1 promoter coupled to luciferase (27). When bovine RPE cells were not stimulated (bar 1) or stimulated with LPS alone (bar 2), no increase of luciferase activity was detected. Stimulation with IFN-γ (bar 3) increased luciferase activity and was increased greatly by the coaddition of LPS in the culture medium (bar 4). The addition of genistein (bar 5) or PDTC (bar 6) largely decreased the luciferase activity induced by LPS/IFN-γ. Furthermore, the LPS/IFN-γ-induced luciferase activity was reduced in cells previously coinubated with PD98059 (bar 7) or with SB 203580 (bar 8) at concentrations that prevented IRF-1 mRNA accumulation. Taken together, these results demonstrated that PDTC and protein kinase inhibitors prevented the induction of IRF-1 gene and the accompanying IRF-1 expression and activity.

**Activation of ERK and p38 MAP Kinase by LPS and IFN-γ in RPE Cells**—To investigate further the association of ERK and p38 MAP kinase activation with NOS-2 induction in RPE cells, serum-starved RPE cells were treated with LPS and IFN-γ alone or combined. The activation of ERK and p38 MAP kinase was determined by Western blot analysis, using antibodies specific for the activated forms of the two kinases. Fig. 7A shows that p38 MAP kinase phosphorylation was observed only after LPS treatment (lane 3) but not after IFN-γ stimulation (lane 2) or in unstimulated cells (lane 1). The coaddition of LPS and IFN-γ induced maximal accumulation of active phosphorylated p38 MAP kinase (lane 4), which was largely prevented by genistein (lane 5) and by SB 203580 (lane 6). In contrast, stimulation of cells with IFN-γ (Fig. 7B, lane 2) or with LPS (lane 3) induced ERK1/2 phosphorylation as noted by the appearance of the characteristic doublet of 42 and 44 kDa, which was absent in unstimulated cells (lane 1). Maximal phosphorylation occurred with the combined stimulation of LPS and IFN-γ (lane 4). This LPS/IFN-γ-induced phosphorylation of ERK1/2 was decreased in the presence of genistein (lane 5). Pretreatment with PD98059 (lane 6), the MEK1 inhibitor, resulted in an inhibition of ERK1/2 activation caused by LPS/IFN-γ.

**DISCUSSION**

We have demonstrated that induction of NOS-2 activity in RPE cells by LPS/IFN-γ implicates a transcriptional mechanism dependent upon the IFN-γ-activated factor, IRF-1, and that transcription of IFR-1 and NOS-2 can be regulated by different MAP kinase pathways and oxidative mechanisms.

Transfection analysis with luciferase-reporter constructs containing the promoter of NOS-2 demonstrated that LPS/IFN-γ-mediated transcriptional regulation of NOS-2 gene expression, explaining in part the accumulation of NOS-2 mRNA after LPS and IFN-γ stimulation (16, 23). In bovine RPE cells, LPS, but not IFN-γ alone, increased murine NOS-2 promoter activity, as reported previously in murine macrophages with
the same promoter (18, 26). However, in contrast to murine macrophages, IFN-γ did not potentiate LPS-induced promoter induction in bovine RPE cells. It could be the result of a species effect because in transfection experiments of this murine promoter in RMG cells from mice, we observed a potentiation of the induction of the promoter by the addition of IFN-γ with LPS. The lack of an IFN-γ effect on the murine-derived constructs in bovine cells suggests the absence of one or more nuclear factors in bovine RPE cells which could not recognize the murine promoter. This incapacity of the murine NOS-2 promoter to respond to IFN-γ in bovine RPE cells is similar to the hyporesponsiveness of the human NOS-2 promoter transfected in murine macrophages to IFN-γ, alone or combined with other cytokines (31, 32).

The inhibition of LPS/IFN-γ-induced NOS-2 promoter activity by genistein and by PDTC, reported previously as inhibitors of NOS-2 mRNA accumulation in RPE cells (16), confirmed the direct involvement of tyrosine kinase and oxidative pathways in the induction of the NOS-2 gene in bovine RPE cells. We have identified some protein kinases involved in the LPS/IFN-γ-induced NOS-2 pathway by using specific antibodies and specific inhibitors of MAP kinases. We demonstrated that ERK1/2, described previously as a target of growth factors in RPE cells (33, 34), was also activated by the inducers of NOS-2 (LPS and IFN-γ) and that LPS was also able to activate p38 MAP kinase. The inhibition of LPS/IFN-γ-induced luciferase activity related to the NOS-2 promoter construct and of LPS/IFN-γ-induced NO production by specific inhibitors of either the ERK1/2 pathway (PD98059) or the p38 MAP kinase pathway (SB 203580) suggested the participation of these two MAP kinase pathways in the induction of NOS-2, as described recently in brain astrocytes and microglial cells (35). In cardiomyocytes, interleukin-1β/IFN-γ-induced NOS-2 mRNA synthesis also involved activation of the ERK pathway (36), whereas in DLD-1 cells neither ERK1/2 nor p38 MAP kinase was required for the regulation of NOS-2 mRNA by IFN-γ/tumor necrosis factor-α (37). These differences reflect probably the cell type-, species-, and stimuli-specific regulation of the NOS-2 gene.
FIG. 7. Western blot of phosphorylated p38 MAP kinase (panel A) and ERK1/2 (panel B). Serum-starved RPE cells were treated for 15 min without IFN-γ (lanes 1) or with 100 units/ml IFN-γ (lanes 2), 1 μg/ml LPS (lanes 3), LPS/IFN-γ (lanes 4), LPS/IFN-γ with 90 μg genistein (lanes 5), or LPS/IFN-γ with either 25 μM SB 203580 (lane 6 in panel A) or 10 μM PD98059 (lane 6 in panel B). When used, protein kinase inhibitors were added to the cells 2 h before stimulation. After 15 min of stimulation, cells were lysed and subjected to immunoblot analysis using antibodies specific for the active p38 MAP kinase (panel A, top) or for the active ERK kinases (panel B, top), as described under “Materials and Methods.” Parallel blots were run, using the antibodies recognizing the total p38 MAP kinase (panel A, bottom) or total ERK2 (panel B, bottom). In each case the experiment shown represents one of three independent blots that gave identical results.

The sensitivity of LPS/IFN-γ-induced NOS-2 mRNA accumulation to cycloheximide (16) and the transcriptional activation of IRF-1 after IFN-γ stimulation (23) indicate the importance of this factor in the induction of NOS-2 in RPE cells, as demonstrated previously in other cell types (21, 22, 38–40). Reverse transcriptase–PCR analysis confirmed that IRF-1 mRNA is synthesized de novo in contrast to IRF-2, its repressor constitutively expressed in RPE cells (23). Because these two factors are similar but apparently compete for the same cis-acting recognition sequences, leading to opposite effects on gene transcription (30), the accumulation of IRF-1 and the decrease of IRF-2 after LPS/IFN-γ treatment could favor the interaction of IRF-1 with the NOS-2 promoter, resulting in an activation of NOS-2 gene transcription. Furthermore, we demonstrated by Western blot and EMSA that IFN-γ-induced IRF-1 protein accumulation and the formation of DNA-IRF-1 binding sequence complexes. These effects of IFN-γ are attributable to activation of the IRF-1 gene by IFN-γ in RPE cells because it was able to increase luciferase activity in transfection experiments with the IRF-1 promoter constructs. Furthermore, LPS, which is required for NOS-2 induction, slightly potentiated IFN-γ-induced IRF-1 gene transcription and the consecutive IRF-1 activation but had no effect by itself on the induction of IRF-1. This suggests an effect of LPS on the induction of the IRF-1 promoter only when cells are already stimulated with IFN-γ, as suggested in HepG2 cells transfected with the same plasmids, where tumor necrosis factor–α, ineffective by itself, largely potentiated the IFN-γ response (27).

By using the nonspecific tyrosine kinase inhibitor, genistein, we demonstrated that induction of the IRF-1 gene and IRF-1 activation required the action of tyrosine kinases. This phenomenon could be explained by an inhibition of the phosphorylation of one component of the JAK/STAT pathway, a transductional signal already described for the IFN-γ (14). As concerns the MAP kinases, our results with specific inhibitors demonstrated that ERK1/2 and p38 MAP kinase are partially involved in IRF-1 induction. However, because MAP kinases are required for IRF-1 induction, they are not sufficient since LPS that activated ERK1/2 and p38 MAP kinase had no effect on IRF-1 promoter activity. An additional tyrosine kinase-dependent step, such as involves JAK kinases, suggested above, could be necessary for IRF-1 induction. Furthermore, we have demonstrated that the antioxidant PTDTC, which blocked NOS-2 promoter activation (Fig. 1) and NOS-2 mRNA accumulation (16), is also able to prevent IRF-1 promoter activation, IRF-1 mRNA and protein accumulation, and IRF-1-DNA interactions. The presence of consensus sequences for NF-κB in the IRF-1 promoter (27) could be responsible for this redox-sensitive step in IRF-1 gene transcription. An important finding in the present study is that although IFN-γ alone induced binding of IRF-1, it failed to induce NOS-2 mRNA and nitrite production. However, inhibition of LPS/IFN-γ-induced IRF-1 activation partially prevented NOS-2 induction, demonstrating that induction of IRF-1 is required to induce NOS-2 in bovine RPE cells. In this context, RPE cells are very similar to other cells types, such as macrophages (21, 22) or islet cells (40), where IRF-1 has been reported to be necessary, but not sufficient, for the induction of NOS-2.

Our study suggests the existence of different intracellular signaling pathways in NOS-2 induction in RPE cells because tyrosine kinase inhibitors blocked NOS-2 mRNA accumulation, without affecting NF-κB binding (16) but inhibiting the induction of IRF-1. This suggests that for NOS-2 expression, two factors, NF-κB and IRF-1, are necessary but not sufficient alone, and their activation or induction implicates a redox-sensitive process, tyrosine kinases and MAP kinases.
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9730–9734
27. Pine, R. (1997) *Nucleic Acids Res.* 25, 4346–4354
28. Renaud, F., Desset, S., Oliver, L., Gimenez-Gallego, G., Van Obberghen, E., Courtois, Y., and Laurent, M. (1996) *J. Biol. Chem.* 271, 2801–2811
29. Goureau, O., Bellot, J., Thillaye, B., Courtois, Y., and de Kozak, Y. (1995) *J. Immunol.* 154, 6518–6523
30. Harada, H., Fujita, T., Miyamoto, M., Kimura, Y., Maruyama, M., Furia, A., Miyata, T., and Taniguchi, T. (1989) *Cell* 58, 729–739
31. Spitsin, S. V., Koprowski, H., and Michaels, F. H. (1996) *Mol. Med.* 2, 226–235
32. Zhang, X., Lauthbach, V. E., Alley, E. W., Edwards, K. A., Sherman, P. A., Russell, S. W., and Murphy, W. J. (1996) *Exp. Cell Res.* 239, 11–15
33. Hinton, D. R., He, S., Graf, K., Yang, D., Hsueh, W. A., Ryan, S. J., and Law, R. E. (1998) *Exp. Cell Res.* 239, 11–15
34. Guillonneau, X., Bryckaert, M., Launay-Longo, C., Courtois, Y., and Mascarelli, F. (1998) *J. Biol. Chem.* 273, 22367–22373
35. Bhat, N. R., Zhang, P., Lee, J. C., and Hogan, E. L. (1998) *J. Neurosci.* 18, 1633–1641
36. Singh, K., Balligand, J., Fischer, T. A., Smith, T. W., and Kelly, R. A. (1996) *J. Biol. Chem.* 271, 1111–1117
37. Kleinert, H., Eichenhofer, C., Fritz, G., Ihrig-Biedert, I., and Forstemann, U. (1998) *Br. J. Pharmacol.* 123, 1716–1722
38. Hecker, M., Preiss, C., Klemm, P., and Busse, R. (1996) *Br. J. Pharmacol.* 118, 2178–2184
39. Fujimura, M., Tominaga, T., Kato, I., Takasawa, S., Kawase, M., Taniguchi, T., Okamoto, H., and Yoshimoto, T. (1997) *Brain Res.* 759, 247–250
40. Flodstrom, M., and Eizirik, D. L. (1997) *Endocrinology* 136, 2747–2753