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Control of Nitric Oxide Production by Endogenous TNF-α in Mouse Retinal Pigmented Epithelial and Muller Glial Cells[1]

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Since the induction of nitric oxide synthase (NOS) by lipopolysaccharide (LPS) has been suggested to be partially dependent of the synthesis of tumor necrosis factor α (TNFα), we have investigated in vitro the production of NO in retinal cells from mice deficient in Lymphotoxin α (LTα)/TNFα. Treatment of retinal Müller glial (RMG) and retinal pigmented epithelial (RPE) cells from both wild-type and knockout mice with LPS and interferon γ (IFNγ) induced NO synthesis as determined by nitrite release into the media and was correlated to an increase in NOS-2 mRNA levels, evaluated by RT-PCR. However, the level of nitrite and the accumulation of mRNA was always less in cells from LTα/TNFα knockout mice than in wild-type mice. Simultaneous addition of TNFα restored the level of NO synthesis by RMG and RPE cells from LTα/TNFα knockout mice stimulated with LPS and IFNγ to wild type levels. Transforming growth factor β (TGFβ) blocked LPS/IFNγ-induced NO production in RMG and RPE cells from wild-type and LTα/TNFα knockout mice. Our results demonstrate that induction of NO synthesis in RMG and RPE cells by LPS and IFNγ is dependent in part on endogenous TNFα while inhibition of NO production by TGFβ does not require a modulation of TNFα synthesis. © 1997 Academic Press

Nitric oxide (NO), a free radical gas, is synthesized from L-arginine by the action of NO synthase (NOS), an NADPH-dependent enzyme (1,2). Among the different isoforms of NOS, a transcriptionally inducible form (iNOS or NOS-2), has been described in different cell types and particularly studied in macrophages (2). NO produced in high concentrations by the inducible form NOS-2 can mediate antimicrobial processes in activated cells (2) but may be also involved in some pathological states (3). In the retina, cultured retinal pigmented epithelial (RPE) cells and retinal Müller glial (RMG) cells stimulated by different combinations of lipopolysaccharide (LPS) and cytokines, such as interferon γ (IFNγ), tumor necrosis factor α (TNFα) and interleukin-1β, express the mRNA for NOS-2 and release nitrite, the stable end product of NO, into the culture supernatant (4-8).

Numerous cytokines and microbial products such as LPS act in synergy to induce the expression of NOS-2. The effective agents and combinations depend on cell type and species (1,9). In this context, TNFα is generally not able to induce NO production by itself, but is believed to increase the induction of NOS-2 expression by microbial products and/or cytokines through an autocrine loop (9). In murine macrophages, the endogenous production of TNFα has been shown to participate to NO production following stimulation by muramyl dipeptide (MDP) and IFNγ (10). Furthermore, monoclonal antibody specific for TNFα blocked IFNγ-induced NO release in macrophages from mice infected by *Leshmania* or *Toxoplasma* (11,12). It has been recently reported that cultured murine RPE and RMG cells are able to express TNFα mRNA and can release TNFα after LPS and LPS/IFNγ stimulation (13-16).

In the present study, we have used RMG and RPE cells from LTα/TNFα deficient mice (17) to investigate the role of endogenous TNFα in the induction of NOS-2 by LPS and IFNγ treatment.

MATERIALS AND METHODS

Mice and cell cultures. The LTαTNFα - mutation inactivates both genes and has been described (17, 18). LTαTNFα $^{+/+}$ and LTαTNFα $^{-/-}$ mice of both sexes were bred on a mixed 129Sv × C57Bl/6 back-

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Abbreviations: NO, nitric oxide; NOS, nitric oxide synthase; RMGcells, retinal Müller glial cells; RPE cells, retinal pigmented epithelial cells.

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ground (17). RMG and RPE cells were cultured from wild-type and knock-out mice at the age of 9-11 days postnatal (14,19). Primary cultures and early subcultures (up to three passages) were used.

Chemicals, cytokines and antibodies. LPS from *Salmonella typhimurium* was obtained from Sigma France. Mouse recombinant IFNγ and human recombinant TNFα were obtained from Pepro Tech Inc. (TEBU, Le Perray en Yvelines, France). TGFβ was purchased from R&D Systems (Abingdon, UK).

Assay for nitrite determination. Confluent RMG and RPE cells in 12-well plates were treated with the indicated combinations of LPS, IFNγ, TNFα and TGFβ in fresh DMEM / 10% FCS. After a 48 h incubation, nitrite concentration was determined in cell-free culture supernatants using the spectrophotometric method based on the Griess reaction, as previously described (4).

RNA isolation and RT-PCR analysis. Total RNA was extracted from activated cultured cells by lysis in guanidinium isothiocyanate followed by phenol acid extraction (20). One μg RNA was reverse transcribed for 90 min. at 42°C with 200 U of superscript Moloney Murine Leukemia virus reverse transcriptase (Life Technologies SARL, Eragny, France), using random hexamers, and 2 μl of cDNA was added to each PCR reaction, as previously described (21). Amplification was performed as follows: 94°C for 2 min.; 28 cycles for NOS-2 and 22 cycles for GAPDH (these number of cycles were to chosen to ensure that the amplification had not reach a plateau) at 94°C for 30 sec, 55°C for 30 sec, 72°C for 45 sec; and then 72°C for 2 min. The amplified fragments were separated on a 1.2% agarose gel and transferred onto a Nylon membrane (Amersham, Les Ulis, France). Identity of the amplification product was confirmed by hybridization with ^{32}P-labeled specific internal oligonucleotide probe, washed 3 times in 1×SSC, 0.1% SDS at 50°C and exposed to X-ray films. The nucleotide sequences of the oligonucleotide primers specific for mouse NOS-2 and GAPDH used for RT-PCR and that of hybridization probes are as follows: NOS-2 antisense (TGTGTCTGCAGATGTGCTAAAC), NOS-2 sense (TTTCTCTTCAAAGTCAAATCCTACCA), NOS-2 hybridization probe (GGGTCGATGTCACATGCAGCTTGTCCAGGGA), GAPDH antisense (ATGGCATGGACTGTGGTCAT), GAPDH sense (ATGCCCCATGTTTGTGATG), GAPDH hybridization probe (GCTGACAATCTTGAGGGAGTTGTCATATTT).

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

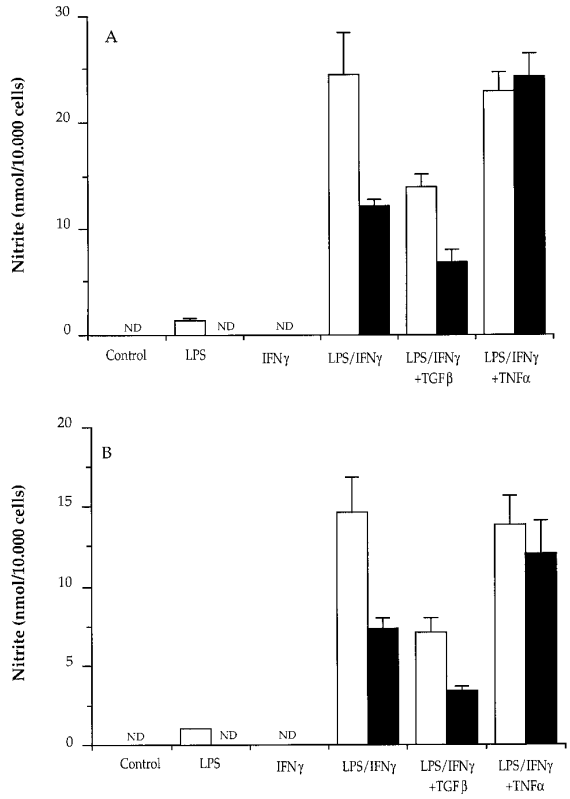

FIG. 1. Nitrite level in supernatants of stimulated RMG and RPE cells from wild-type and LTα/TNFα knockout mice. RMG cells (A) or RPE cells (B), from wild-type (open columns) and from LTα/TNFα knockout mice (black columns), were incubated with LPS (1μg/ml), IFNγ (100U/ml), TGFβ (5ng/ml), or TNFα (100U/ml) as indicated. After 48 hours, nitrite level was measured in the culture medium using the Griess reagent. Values are means ±S.E.M. from three independent cultures.

RESULTS

In the mouse RMG cells from wild-type mice, a slight increase in nitrite synthesis was found after LPS treatment, while IFNγ alone was unable to induce nitrite release (Figure 1A). Maximal nitrite release was obtained when LPS was associated with IFNγ. Similarly, in the RPE cells from wild-type mice, nitrite production was slightly induced by the presence of LPS but not by IFNγ alone as previously demonstrated (7), while an important potentiation was observed when both stimuli were used (Figure 1B). Coincubation of RMG or RPE cells from wild-type mice with TGFβ and LPS/IFNγ for 48 hours reduced nitrite release (Figure 1).

We examined whether endogenous TNFα could regulate the capacity of RMG and RPE cells to produce NO after LPS and IFNγ stimulation. After stimulation with LPS and IFNγ, RMG and RPE cells from LTα/TNFα knockout mice produced approximately 40% less nitrite than wild-type cells (Figure 1). Addition of TNFα concurrently with LPS and IFNγ restored NO synthesis to the levels observed with wild type RMG and RPE cells (Figure 1), confirming the involvement of TNFα in the pathway of LPS/IFNγ-induced nitrite production. Interestingly, in RMG and RPE cells from LTα/TNFα knockout mice, no nitrite could be detected when LPS was added alone, in contrast to cells from wild-type mice (Figure 1). Furthermore, the addition of TGFβ to the culture medium of LTαTNFα −/− RMG or RPE cells always inhibited the production of nitrite induced by LPS and IFNγ as efficiently as in wild type cultures (Figure 1).

The induction of NO production by LPS and IFNγ in retinal cells involves an increase in NOS-2 mRNA accumulation (5,22). An RT-PCR analysis was performed to determine whether endogenous TNFα also acted at the level of NOS-2 mRNA accumulation. RNA were prepared from RMG and RPE cells of both genotypes after 24 hours of stimulation, which the time at which NOS-2 mRNA accumulation is maximal (5,22). In samples from wild type RMG and RPE cells stimu-

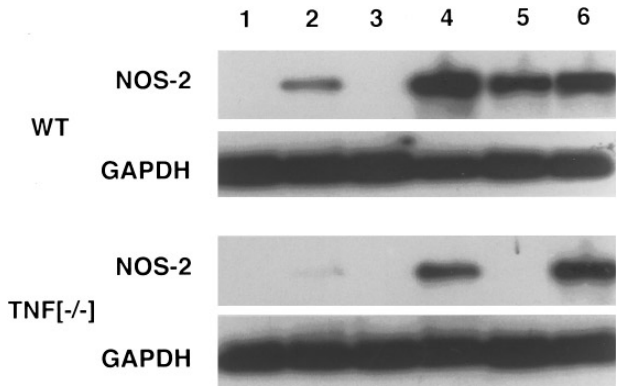

FIG. 2. Decrease of NOS-2 mRNA accumulation in activated RMG cells from LTα/TNFα knockout mice. Confluent RMG cells from wild-type and LTα/TNFα knockout mice were stimulated for 24 hours with: no stimulation (1), 1μg/ml LPS (2), 100U/ml IFNγ (3), LPS and IFNγ (4), LPS/IFNγ plus 5ng/ml of TGFβ (5) or LPS/IFNγ plus 100U/ml of TNFα (6).Total RNA were extracted and the levels of NOS-2 and GAPDH mRNAs were assessed by RT-PCR analysis. The results presented comes from one of three independent experiments which gave similar results.

lated with LPS and IFNγ, NOS-2-specific primers generated a PCR product of the expected size of 650 bp, which hybridized with a ^{32}P-labeled internal oligonucleotide probe (figures 2 and 3). In the unstimulated RMG cells (figure 2) and RPE cells (figure 3), no signal was detectable. As observed for nitrite release, LPS alone was able to induce NOS-2 mRNA accumulation in RMG and RPE cells. In contrast IFNγ alone had no effect in RMG (figure 2) and in RPE cells (data not shown). No significant difference in the expression of GAPDH PCR product at 169 bp, used as an internal control, was observed. RMG and RPE cells from LTα/TNFα knockout mice expressed lower levels of NOS-2 mRNA than wild-type cells, after LPS or LPS/IFNγ stimulation (Figure 2 and 3), in accordance with the NOS activity previously evaluated by nitrite release. The presence of TGFβ decreased the accumulation of NOS-2 mRNA in RMG and RPE cells stimulated with LPS/IFNγ, from both wild-type and LTα/TNFα knockout mice.

DISCUSSION

In this report, we demonstrate that LPS and LPS/IFNγ-induced NO synthesis in mouse RMG and RPE cells is partially dependent on the endogenous production of TNFα. Nitrite release was reduced in RMG and RPE cells from LTα/TNFα deficient mice and addition of TNFα concurrently with LPS and IFNγ restored NO synthesis to the same level as RMG and RPE cells from wild-type mice. Thus the reduction of NO synthesis due to the LTα/TNFα deletion can be totally corrected by addition of exogenous TNFα which confirms the involvement of TNFα in NOS induction by LPS/IFNγ in mouse retinal cells. Although TNFα is the prime candidate for being the actual mediator, we cannot exclude that LTα could be also involved as these molecules share many biological activities. The decrease of nitrite release in LTα/TNFα knockout mice correlated well with the decrease of NOS-2 mRNA expression, suggesting a direct effect of TNFα on NOS-2 gene transcription. In accordance with this hypothesis, consensus sequences have been for transcription factors activated by TNFα have been identified in the promoter of NOS-2 gene (23,24). Our results are in agreement with earlier published experiments (25) that demonstrated, by using TNF receptor-1 $-/-$ mice, that nitrite release induced by LPS and IFNγ is reduced in macrophages from these mice infected with *mycobacterium tuberculosis* compared to infected wild type mice. More recently TNF receptor 1-mediated signaling has been also involved in the *in vivo* induction of NOS-2 mRNA by LPS in liver but not in spleen (26), suggesting an organ specific in vivo regulation of NOS-2 mRNA expression. In this context, our results demonstrate that retina **is** a tissue where NOS induction by LPS is dependent in part on an endogenous TNFα synthesis.

We confirmed in this study that TGFβ inhibits the production of nitrite in mouse RMG and RPE cells, as previously reported (5-7). The inhibitory effect of TGFβ correlated with the decrease of NOS-2 mRNA accumulation. Inhibition of NO synthesis was unaffected by the absence of endogenous TNFα, since TGFβ inhibited NOS-2 mRNA accumulation and nitrite release in cells from LTα/TNFα deficient mice. These results indicate that inhibition of NO production by TGFβ does not operate at the level of TNFα synthesis. Since TNFα

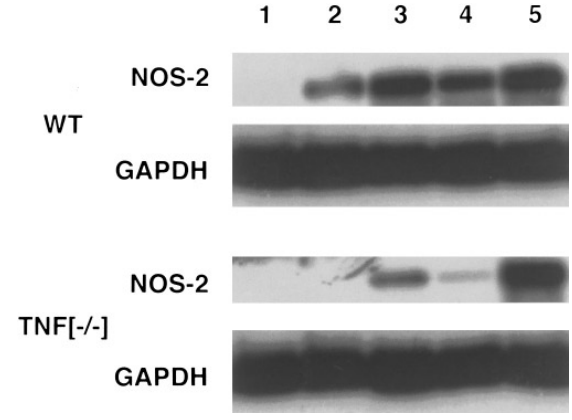

FIG. 3. Decrease of NOS-2 mRNA accumulation in stimulated RPE cells from LTα/TNFα knockout mice. Confluent RPE cells from wild-type and LTα/TNFα knockout mice were stimulated for 24 hours with no stimulation (1), 1μg/ml LPS (2), LPS and IFNγ (3), LPS/IFNγ plus 5ng/ml of TGFβ (4), or LPS/IFNγ plus 100U/ml of TNFα (5). Then total RNA were extracted and the levels of NOS-2 and GAPDH mRNAs were assessed by RT-PCR analysis. The results presented come from one of two independent experiments which gave similar results.

and NO are involved in ocular inflammation, in tissue necrosis and regulate interactions between immune and retinal resident cells (21, 27-29), it would be interesting to test the ability of LTα/TNFα deficient mice to develop ocular pathologies after different experimental treatment, such as LPS injection.

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