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DIFFERENTIAL REGULATION OF CONSTITUTIVE AND INDUCIBLE NITRIC OXIDE PRODUCTION BY INFLAMMATORY STIMULI IN MURINE ENDOTHELIAL CELLS

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The murine vascular endothelial cell line send1 expresses both constitutive and inducible nitric oxide (NO) synthases. Interferon-gamma (IFNγ) or endotoxin (LPS) alone inhibited constitutive NO production in a dose and time dependent manner. Addition of L-arginine had no influence on the decrease of NO production caused by IFNγ or LPS. On the other hand, IFNγ and LPS synergized in the induction of high output NO production. Successive incubations with IFNγ and LPS in different sequences revealed IFNγ as the time setting signal for the induction of NO synthesis. These results demonstrate that LPS and IFNγ have a direct suppressive effect on constitutive NO synthase while at the same time they strongly activate inducible NO production. Thus inflammatory stimuli trigger murine vascular endothelial cells to switch from constitutive to inducible NO synthase activity.

Nitric oxide (NO), formed from the guanidino nitrogen of L-arginine, mediates vasodilatation through activation of the soluble guanylat-cyclase in vascular smooth muscle cells (1). Two isoforms of NO synthase (NOS) have been characterised: a constitutive, calcium/calmodulin dependent (cNOS) and a cytokine-inducible, calcium/calmodulin independent form (iNOS; for review 2). Besides numerous other cell types, NO is produced by vascular endothelial cells, that play a central role in the control of vascular tone under normal and pathophysiologic conditions and are a target for cytokines (3, for review 4). Activation and deactivation of inducible NO production by cytokines have been described in murine macrophages (5) and endothelial cells (6).

There is only scant knowledge about the effects of cytokines on constitutive NO production. In the present study we investigated the effects of the inflammatory stimuli LPS and IFNγ on the constitutive and inducible NO production by murine vascular endothelial cells. Our study provides evidence for a differential regulation of constitutive and inducible NO production by inflammatory stimuli within a single cell type disposing of both cNOS and iNOS.

ABBREVIATIONS: IFNγ, interferon-gamma; LPS, endotoxin; NO, nitric oxide; NOS, nitric oxide synthase.

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MATERIALS AND METHODS

Cell culture. The murine vascular endothelial cell line send1 (7), kindly provided by Dr. K. Ballmer, Friedrich Miescher Institute (Basel, Switzerland), was maintained in a humidified 5% CO_2/95% air atmosphere at 37° C in IMDM (Gibco Europe, Basel, Switzerland) supplemented with 10% fetal calf serum (low tox, batch no. 01530/901, PAA, Linz, Austria), 4 mM L-glutamine and 50 µg/ml gentamycin (both Sigma Chemical Co., St. Louis, MO, USA), referred to as complete medium. For some experiments, complete medium was supplemented with 500 µM L-arginine (tissue culture grade, Sigma). Cells were seeded in 6-well cluster plates (Falcon, Oxnard, CA, USA) and used for experiments when they reached confluence.

Chemicals. Murine recombinant IFNγ was purchased from Genzyme (Cambridge, MA, USA). Endotoxin level: 0.001 ng/µg IFNγ (determined by a kinetic-chromogenic method by the manufacturer). LPS (E.coli O26:B6, Boivin extraction) was from Difco (Detroit, MI, USA).

Measurement of nitric oxide/nitrite. Nitrite in supernatant was measured by the Griess method as described (8). Sodium nitrite diluted in complete medium was used as standard. The detection limit for NO_2^- was 0.1 µM/10^6 cells.

Determination of cell numbers and viability. Cells were counted electronically after stromatolysis with Zap-oglobin (Coulter Electronics, Luton UK) in a Coulter model ZBI counter. Cell viability was assessed by trypan blue exclusion and was >95% throughout the experiments.

Statistics. Results are given as mean ± SD of triplicate wells and duplicate measurements. Statistical analysis was made by Dunnett's multiple comparison test with significance set at $p<0.05$.

RESULTS AND DISCUSSION

Effects of LPS and IFNγ on constitutive and inducible NO production: basic observations. Murine endothelial cells constitutively produced NO when untreated (Fig. 1). Incubation for 48h with LPS (1 µg/ml) or IFNγ (100 U/ml) significantly suppressed

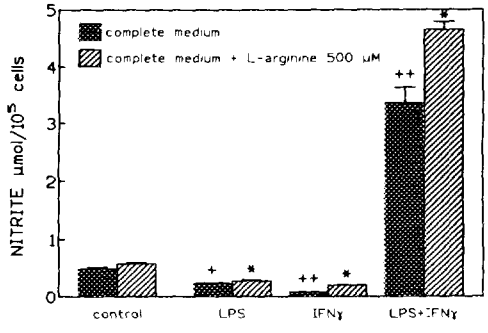


FIGURE 1.
Effects of IFNγ and/or LPS on NO production. Confluent cells were incubated for 48h without stimulus (control), with IFNγ (100 U/ml) and/or LPS (1 µg/ml) as indicated. Values are means ± SD from triplicate wells and duplicate measurements of one representative out of three independent experiments. +, $p<0.05$, ++, $p<0.01$ as compared to control in complete medium; *, $p<0.01$ as compared to control in complete medium with L-arginine.

this constitutive NO production. The inhibitory effect was not due to substrate depletion as observed in murine macrophages (9), since substitution with L-arginine did not alter the inhibition, although slightly more NO was produced when L-arginine was added. Combined LPS/IFNγ induced murine endothelial cell NO production. L-arginine substitution further enhanced induced NO synthesis indicating that intracellular availability of substrate is limiting.

Effect of LPS on constitutive NO synthesis. As shown in Fig. 2A, LPS inhibited constitutive NO production by murine vascular endothelial cells cultured for 48h in complete medium in a dose dependent manner, with a threshold effect observed at 1 ng/ml LPS. After 6h of incubation, the amount of nitrite in the culture supernatant was still at the level of NO produced by untreated cells, whereas 12h after addition of LPS a decrease could be detected (Fig. 3). Although NO production was higher, the percentage of inhibition remained unaffected when the medium was supplemented with 500 μM L-arginine, the substrate for NOS (Figs. 2A and 3), suggesting a direct effect of LPS on NO production.

Effect of IFNγ on constitutive NO production. Incubation of send1 cells for 48h with IFNγ led to a concentration-dependent decrease of the basal NO output (Fig. 2B). 10 U/ml IFNγ was necessary to evoke the inhibitory effect. Substitution of the culture medium with 500 μM L-arginine significantly diminished, but did not abolish the inhibition of NO production by IFNγ (Fig. 2B). Similarly to LPS, IFNγ required more than 6h to establish its inhibitory effect (Fig 5; filled squares).

Effects of combined LPS/IFNγ on inducible NO production. Fig. 4 shows that more than 50 U/ml IFNγ were required to induce NO production when given simultaneously with LPS, a concentration about 5 times higher than the dose of IFNγ needed for

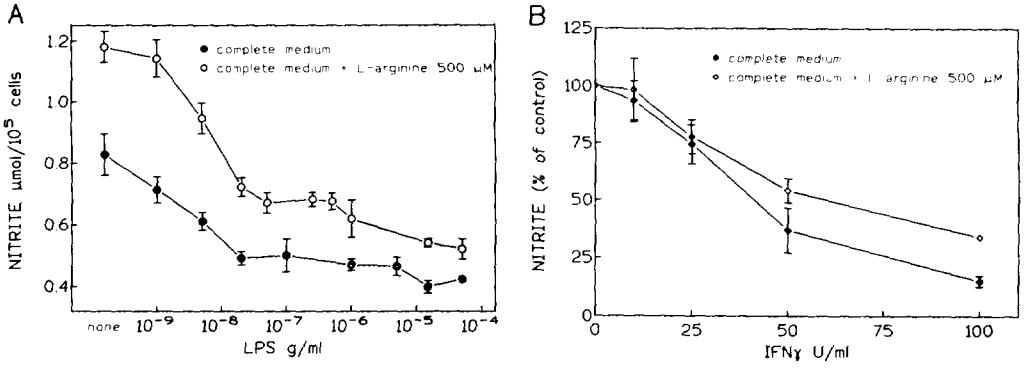
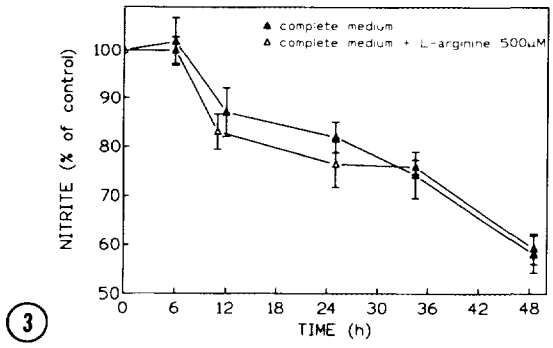


FIGURE 2.

Dose-response of the inhibitory effect of LPS or IFNγ on constitutive NO production. Confluent cells were incubated for 48h with increasing concentrations of either LPS (A) or IFNγ (B) as indicated, in the presence or absence of L-arginine. Mean values ± SD from triplicate wells and duplicate measurements of one representative experiment are shown.



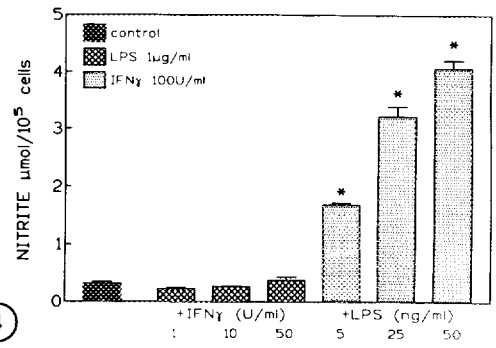


FIGURE 3.
Time-course of the LPS induced inhibition of constitutive NO production. Confluent cells were incubated with 1 µg/ml LPS in complete medium with or without 500 µM L-arginine as indicated. After every measurement, culture medium was removed and replaced. Presented are means ± SD from triplicate wells and duplicate measurements from one out of three identical experiments.

FIGURE 4.
Alteration of NO production by combinations of LPS with IFNγ. Confluent cells were incubated for 48h in complete medium supplemented with 500 µM L-arginine with either a fix concentration of LPS (1 µg/ml) combined with increasing IFNγ or a fix dose of IFNγ (100 U/ml) combined with increasing concentrations of LPS, as indicated. Shown are means ± SD from triplicate wells and duplicate measurements from one representative experiment. *, $p<0.01$ as compared to control.

inhibition of constitutive NO production when given alone. However, LPS concentrations in the same range as necessary for an inhibitory effect upon constitutive NO output (when given alone) sufficed for the synergistic stimulation of NO production in combination with IFNγ. When added simultaneously with 100 U/ml IFNγ, LPS dose-dependently induced NO synthesis (Fig. 4). Highest amounts of nitrite were produced after induction with 100 ng/ml LPS, higher concentrations of LPS did not further enhance NO production (data not shown).

Effect of sequential incubations with LPS and IFNγ on inducible NO production. Exposure of send1 cells to IFNγ for 6h followed by addition of LPS (Fig. 5; filled diamonds) had the same synergistic effect on inducible NO production as an incubation with combined LPS and IFNγ from the beginning of the experiment (asterisks): in both cases, increase of nitrite concentrations in the supernatant required 12h. However, when cells were pre incubated for 6h with LPS, followed by addition of IFNγ, a stimulation of NO production could be found only after 18h of incubation (empty diamonds). The same observations were made when cells were pre incubated for 12h with single stimuli: exposure to IFNγ for 12h followed by addition of LPS (filled circles) led to an acceleration of induced NO production compared to the NO output of cells that were first exposed to LPS followed by addition of IFNγ (unfilled circles). Therefore we conclude that IFNγ is the time setting signal to trigger induction of NO synthesis by combined LPS and IFNγ.

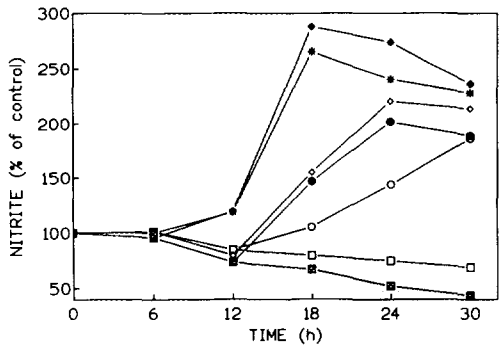


FIGURE 5.
Incubation of NO production by sequential addition of LPS and IFNγ: time-course. Confluent cells received fresh complete medium supplemented with 500 μM L-arginine. Then cells were incubated without further stimuli (control), with LPS (1 μg/ml; unfilled squares), with IFNγ (100 U/ml; filled squares), with combined IFNγ and LPS (asterisks) or with different sequences of LPS and IFNγ: 6h IFNγ, then LPS (filled diamonds); 6h LPS, then IFNγ (unfilled diamonds); 12h IFNγ, then LPS (filled circles); 12h LPS, then IFNγ (unfilled circles). Every 6h, 100 μl of culture supernatant was removed from all wells for determination of nitrite. For clarity, only mean values of triplicate wells and duplicate measurements are presented from one representative experiment. SD are between 5 to 10% of the mean values.

Our results demonstrate that in murine vascular endothelial cells expressing both NOS isoforms, the same inflammatory stimuli exhibit different effects on constitutive or inducible NO synthesis. There is a direct suppressive effect of LPS or IFNγ on the constitutive NO synthesis, that cannot be counterbalanced by additional L-arginine (Figs. 1, 2A and B, 3). Also tetrahydrobiopterin, the obligate cofactor and regulator of NOS activity, is not at limiting levels (manuscript in preparation), and can therefore not account for the inhibition of NO production caused by LPS or IFNγ. We suggest that murine endothelial cells switch from constitutive to inducible NO synthase activity under inflammatory conditions. This event seems not to be restricted to the cell line studied here, but inhibition of cNOS activity by LPS has been described also in bovine endothelial cells (10), disposing of both cNOS and iNOS. In human endothelial cells, which express only cNOS, inflammatory cytokines have been shown to decrease the mRNA for cNOS (11,12). In accordance with findings of others (13,14,15) are our observations that IFNγ in combination with other immunoactivators, but not alone, can stimulate NO production in endothelial cells expressing the gene for iNOS. It must be noted, that trace amounts of LPS are sufficient to synergize with IFNγ at an optimal dose of 100 U/ml to induce high output NO synthesis in murine vascular endothelial cells (Fig. 4).

Because in man cNOS of vascular endothelial cells is suppressed at the transcriptional level by inflammatory stimuli (11,12), and human endothelial cells do not dispose of iNOS (11), the switch from constitutive to inducible NO production under septic conditions involves in man an other cell type, possibly the vascular smooth muscle cell. Therefore in

man, the role of NO as an endothelium-derived relaxing factor has to be reconsidered in inflammatory or septic conditions.

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