Tetrahydrobiopterin-dependent Formation of Nitrite and Nitrate in Murine Fibroblasts

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Summary

The present study demonstrates that murine dermal fibroblasts produce nitrite (NO_2^-) and nitrate (NO_3^-) upon treatment with interferon γ (IFN-γ). This formation is dependent on L-arginine and can be inhibited by the L-arginine analogue N^G-monomethyl-L-arginine. The effect of IFN-γ is drastically increased by cotreatment with tumor necrosis factor α (TNF-α), interleukin 1 (IL-1), or lipopolysaccharide (LPS). The tested cytokines also induce formation of tetrahydrobiopterin in murine fibroblasts. Inhibition of guanosine triphosphate–cyclohydrolase I, the key enzyme of tetrahydrobiopterin de novo synthesis with 2,4-diamino-6-hydroxy-pyrimidine, leads to decreased formation of NO_2^- and NO_3^-. This effect can be reversed by addition of sepiapterin, which provides tetrahydrobiopterin via a salvage pathway. Methotrexate, which inhibits the salvage pathway, blocks the restoration of NO_2^- and NO_3^- production by sepiapterin. The cytotoxic effect of combinations of IFN-α with TNF-γ, IL-1, or LPS is attenuated by inhibition of tetrahydrobiopterin synthesis. These results show that intracellular concentrations of tetrahydrobiopterin control the amount of NO_2^- and NO_3^- produced in situ and suggest that the role of cytokine-induced tetrahydrobiopterin synthesis is to provide cells with the active cofactor for production of nitrogen oxides.

One of the metabolic actions of IFN-γ on human cells in vitro is an up to 100-fold stimulation of guanosine triphosphate (GTP)[1]-cyclohydrolase I (EC 3.5.4.16) activity (1). This enzyme cleaves GTP to 7,8-dihydroneopterin triphosphate, thus initiating the formation of 7,8-dihydroneopterin and 5,6,7,8-tetrahydrobiopterin (2). Both compounds can be detected in cell homogenates of IFN-γ treated human macrophages, human fibroblasts, and a panel of human tumor cell lines (1). We showed previously that further enzyme activities required for the formation of tetrahydrobiopterin, i.e., 6-pyruvoyl tetrahydropterin synthase and sepiapterin reductase (EC 1.1.1.153), are not influenced by IFN-γ, but are constitutively present in the cells (3). IFN-γ-induced stimulation of GTP-cyclohydrolase I activity is enhanced by cotreatment with TNF-α, LPS, or dexamethasone. This was demonstrated for human fibroblasts, human macrophages and the monocytic cell line THP-1 (4, 5). In vivo, cytokine-induced stimulation of GTP-cyclohydrolase I is indicated by elevated neopterin excretion, which is related with diseases challenging the cell-mediated immune system (6, 7).

Synthesis of tetrahydrobiopterin was also observed in murine macrophages (8, 9). Further, we found recently in mouse dermal fibroblasts that tetrahydrobiopterin synthesis is stimulated by IFN-γ and even better by TNF-α, or combination of IFN-γ with TNF-α (Werner, E. R., G. Werner-Felmayer, D. Fuchs, A. Hausen, G. Reibnegger, J. J. Yim, and H. Wachter, manuscript submitted for publication).

Although cytokine-induced pteridine synthesis thus seems to be a general phenomenon in mammalian cells, no biological function could so far be established. Tetrahydrobiopterin is an essential cofactor of phenylalanine 4-, tyrosine 3-, and tryptophan 5-monooxygenase (10). However, we could not find that these enzyme activities were induced in IFN-γ-treated cells (unpublished data). Moreover, there is no evidence that oxidation of etherlipids, the fourth established tetrahydrobiopterin-dependent reaction (11), is influenced by IFN-γ.

Recently, a new tetrahydrobiopterin-requiring reaction, the formation of nitrogen oxides from L-arginine, was described for cell homogenates of mouse macrophages (8, 9). This oxidation of L-arginine is induced in murine macrophages by treatment with LPS (12) or lymphokines, such as IFN-γ (13). The effect of IFN-γ can be further enhanced by cotreatment with TNF-α, TNF-β, or LPS (14, 15). Since pteridine synthesis is modulated by the same factors, it appeared attractive to hypothesize that cytokines induce formation of tetrahydrobiopterin in order to provide a cofactor for the generation of

[1] *Abbreviations used in this paper:* DAHP, 2,4-diamino-6-hydroxypyrimidine; GTP, guanosine triphosphate; NMA, N^G-monomethyl-L-arginine; NO_2^-, nitrite; NO_3^-, nitrate.

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nitrogen oxides. If this was true, pteridine-dependent formation of nitrogen oxides should not only occur in macrophages but also in other cells with inducible pteridine synthesis, e.g., in fibroblasts.

In this report, we show that murine fibroblasts can be stimulated by IFN-γ to release nitrite (NO_2^-) and nitrate (NO_3^-). The effect of IFN-γ is enhanced by TNF-α, IL-1, or LPS. Data obtained by inhibition and restoration of pteridine synthesis indicate that tetrahydrobiopterin is essential for the formation of NO_2^- and NO_3^-, and that generation of nitrogen oxides is related to cytokine-mediated cytotoxicity.

Materials and Methods

Materials. Mouse rIFN-γ (produced in Chinese hamster ovary cells) was obtained from Holland Biotechnology (Leiden, The Netherlands) and had a specific activity of 10^7 U/mg of protein. Mouse rTNF-α (4×10^7 U/mg protein; produced in *Escherichia coli* was a kind gift of Dr. G.R. Adolph (Bender Co., Vienna, Austria). Human rIL-1-α (10^7 U/mg protein; expressed in *E. coli*) was from Genzyme (Boston, MA). LPS from *E. coli* 055:B5 (phenolic extract), 2,4-diamino-6-hydroxy-pyrimidine (DAHP), and methotrexate were from Sigma Chemical Co. (Munich, FRG). N^G-monomethyl-L-arginine (NMA) was from Calbiochem-Behring Corp. (La Jolla, CA). Pteridines were from Dr. B. Schircks (Jona, Switzerland).

Cell Culture. Murine fibroblasts were kindly provided by Dr. C. Heufler (Department of Dermatology, University of Innsbruck). Cells were established from ear dermis explants of 8–10-wk-old BALB/c mice, which were cultured in RPMI 1640 (Biochrome, Berlin, FRG) containing 10% heat-inactivated FCS (Biochrome), 2 mM L-glutamine, 100 U/ml of penicillin, and 0.1 ng/ml of streptomycin (Sigma Chemical Co.). All cultures were maintained in humidified air containing 5% CO_2 at 37°C with a medium change every week. Nonadherent cells were removed by washing. After 4 wk, cells grown out from the explants were passaged by trypsinization and further propagated by a weekly transfer of 1:3. Cells were determined to be fibroblasts due to morphological features, growth characteristics, and MHC antigen expression. The resulting cell population, forming a monolayer, consisted of multipolar cells, assuming a pavement-like and partly spindle-shaped appearance when confluent, as is characteristic for mouse fibroblasts. Cells ceased to grow when confluent, and no formation of foci was observed. For isolation and culture of cells, no pretreatment of surfaces or supply with growth factors except FCS was provided. Under these standard conditions, only fibroblasts survive in the culture. MHC class I antigen expression of these cells was enhanced two- to threefold by IFN-γ (5–500 U/ml) as compared with untreated controls and untreated control cells, and IFN-γ-stimulated cells were negative for MHC class II antigen. This was reported previously for 3T3 mouse fibroblasts (16). MHC antigen expression was detected by ELISA as performed previously (17). Cells grown in 96-well plates were stained with either undiluted supernatant from 34-1-2S hybridoma (K^d, D^d; American Type Culture Collection, Rockville, MD) or with 20 ng/well of purified antibody reacting with I-A^d (Becton Dickinson & Co., Mountain View, CA) and with a horseradish peroxidase–conjugated second antibody to mouse IgG whole molecule (Sigma Chemical Co.). Cells of passages 10–25 were used for experiments.

Formation of NO_2^- and NO_2^- + NO_3^-. Fibroblasts were seeded at a density of 10^5/ml in 24-well culture plates (Falcon Labware, Oxnard, CA) and grown to confluency with one change of medium on day 4 of culture, using RPMI 1640 (supplemented as described above). On day 8, cells were treated with cytokines and/or other additives. Due to its low content of nitrate, all incubations were carried out in DMEM (Serva, Heidelberg, FRG), supplemented as described above. We tested different doses of DAHP (1–10 mM), sepiapterin (50–400 μM), and methotrexate (10–100 μM). Optimal doses were 5 mM for DAHP, 100 μM for sepiapterin, and 10 μM for methotrexate, and were used throughout all further experiments. Accordingly, other pteridines were used at a concentration of 100 μM. In the case of methotrexate treatment, the medium was supplemented with 40 μM thymine and 100 μM inosine (Fluka, Buchs, Switzerland). All additives were dissolved in complete DMEM, and, where necessary, the pH was adjusted to 7.4. The additives were applied to cells just before cytokines. In the case of cytokine combinations, TNF-α, IL-1, or LPS were added immediately after IFN-γ (i.e., within 1 min), which was essential for optimal stimulation. The final volume per well was 1 ml. NMA was applied to cells 24 h before stimulation with cytokines.

For some experiments, confluent monolayers were treated for 7 h with cultured medium with or without L-arginine (MEM/Earle, Select Amine Kit; Gibco Laboratories, Grand Island, NY), containing serum, glutamine, and antibiotics (see above). After changing the respective medium once again, cells were stimulated with cytokines.

After incubation for 72 h, supernatants were harvested. Monolayers were washed once with warm PBS and were lysed with 0.05 M H_3PO_4 for protein determination. NO_2^- was measured in supernatants as described (18), using the stable Griess-Ilosvay's nitrite reagent obtained from Merck (Darmstadt, FRG). NO_3^- was reduced enzymatically by 0.125 μmol/min of nitrate reductase (EC 1.6.6.2) from *Aspergillus* sp. (Serva), and 2.5 mM nicotinamide adenine dinucleotide phosphate, reduced form, and was then measured as NO_2^- (designated NO_2^- + NO_3^-). Background levels were determined in cell-free controls of culture medium with or without additives and were subtracted for calculation of the total amount of nitrogen oxides formed. Data are presented as micromoles per liter. The detection limit of NO_2^- was 1 μmol/liter. Approximate values in nanomoles per milligram of total cell protein or nanomoles per 10^5 cells can be calculated from the protein values given below.

Determination of Intracellular Biopterin. Fibroblasts were seeded in 25-cm^2 culture flasks (Falcon Labware) at a density of 10^5/ml in 5 ml of complete RPMI 1640 and grown to confluency with one medium change. On day 8, cells were treated with cytokines, DAHP, methotrexate, and sepiapterin in a final volume of 5 ml of complete DMEM (for details, see above). After 72 h, supernatants were collected for detection of NO_2^- and NO_2^- + NO_3^- (see above). Absolute values of nitrogen oxides presented in Table 2 (see Results) are higher than in the other experiments shown. This was due to the use of culture flasks (25 cm^2/5 ml) instead of 24-well plates (2 cm^2/ml), resulting in a different cell to supernatant ratio.

Cells were harvested by trypsinization and washed with PBS. Pellets were resuspended in 250 μl of distilled water, rapidly frozen in liquid nitrogen, and kept at −20°C until oxidation, which was started within 1 h after freezing. Samples were thawed one by one, centrifuged for 2 min at 10,000 *g*, and subjected to oxidation with iodine at acidic or alkaline pH (19). Briefly, 100 μl of cell extracts were mixed with 5 μl of 1 M HCl and 5 μl of 0.1 M KI/I_2 in order to determine the total amount of biopterin, including 5,6,7,8-tetrahydro and 7,8-dihydro derivatives. Another 100 μl of cell extract was mixed with 5 μl of 1 M NaOH and 5 μl of 0.1 M KI/I_2

for destroying the tetrahydro derivative, thus detecting the sum of 7,8-dihydrobiopterin and biopterin. After incubation for 1 h in the dark at room temperature, samples were centrifuged for 2 min at 10,000 *g*, and the supernatant was mixed with 10 μl of 0.1 M ascorbic acid. Samples were stored at −20°C until HPLC analyses (LC 5500; Varian Associates, Inc., Palo Alto, CA). Samples were then extracted with AASP-SCX cartridges (Analytichem International, Inc., Harbor City, CA), and pteridines were directly eluted onto a reversed-phase HPLC column (Lichrosorb, RP-18, 7-μm particle size, Merck) and quantified by fluorescence detection (LS 4, Perkin-Elmer Corp., Beaconsfield, UK), as previously described (20). Values are presented as picomoles per milligram of total cell protein.

Cell Viability Assay. Fibroblasts (10^5/ml) were seeded in 100 μl/well in 96-well plates (Falcon Labware) and grown to confluency, using RPMI 1640 with supplements. We used confluent monolayers in order to limit possible effects of additives on cell growth. On day 8 of culture, monolayers were treated with cytokines, DAHP, and pteridines in DMEM (see above) in a final volume of 100 μl/well for 48–96 h. In some experiments, the influence of L-arginine-deficient medium, of 250 μm NMA, of 10 mM L- or D-arginine, and of NMA plus 10 mM L- or D-arginine was tested. For this purpose, confluent monolayers were pretreated with the mentioned additives 48 h before addition of cytokines in the respective culture medium. After 48–96 h, medium was removed, and cells were washed once with 100 μl/well of warm PBS and fixed then with 5% glutaraldehyde in PBS (100 μl/well) for 10 min. Plates were washed three times with PBS. Fixed cells were stained with 0.05% methylene blue (Merck) for 5 min and washed five times with PBS. Methylene blue, trapped in fixed cells, was solubilized with 100 μl/well of 0.33 M HCl, and OD at 620 nm was measured using a 96-well plate reader (Anthos; Labtec, Salzburg, Austria). Relative cell viability is expressed as percent of untreated control cells. For each tested combination of factors, eight wells per plate were treated in parallel. In comparison with 24-well plates (2 cm^2/ml) used in the other experiments, cytotoxic effects could be observed earlier (i.e., after 72–96 h) in 96-well plates (0.32 cm^2/100 μl) due to a different cell to supernatant ratio. NO_2^- was determined in supernatants of individual wells as described above.

Protein Determination. Protein content of cell extracts was determined according to Bradford (21), modified for 96-well plates, using the protein dye reagent from Bio-Rad Laboratories (Richmond, CA) and BSA (Serva) as protein standard. Individual wells of control cells contained 76.6 ± 7.7 μg (mean of 12 cultures ± SD) of protein, which was not reduced >10% by cytokine treatment for 72 h using 24-well plates and 1 ml of culture medium. Protein content of 10^5 cells was 25.4 ± 2.5 μg (mean of 10 cultures ± SD), so that the final cell number per well was ~3 × 10^5.

Results

Induction of NO_2^- and NO_3^- Formation in Murine Fibroblasts by Cytokines. Murine fibroblasts produced NO_2^- and NO_3^- upon treatment with IFN-γ in a dose-dependent manner (Fig. 1). The effect of IFN-γ was about two- to threefold enhanced by cotreatment with TNF-α, IL-1, or LPS. NO_2^- represented 58.2 ± 6.3% (mean of 36 cultures ± SD) of the total amount of NO_2^- + NO_3^-, as calculated from the values given in Fig. 1. TNF-α, IL-1, or LPS were nearly ineffective as single stimuli. However, in the case of IL-1, 10 U/ml was sufficient to enhance the IFN-γ effect, and 100 U/ml had only a slightly better costimulatory effect (Fig. 1). For LPS, optimal costimulation of 50 U/ml of IFN-γ was achieved at 100 ng/ml. LPS doses higher than 100 ng/ml did not further enhance the effect of IFN-γ. Maximal production of NO_2^- was observed upon treatment of cells with 50 U/ml IFN-γ in combination with 500 U/ml of TNF-α (Fig. 1). This cytokine combination was therefore used in further experiments.

Fig. 2 shows the time course of NO_2^- and NO_2^- + NO_3^- formation upon stimulation with 50 U/ml of IFN-γ in combination with either 500 U/ml TNF-α, 100 U/ml IL-1, or 10 μg/ml LPS. Despite the cytotoxic effects of these cytokines (see below), which can be observed after >72 h, there is a steady increase of NO_2^- and NO_2^- + NO_3^- in the supernatants over 144 h. Protein content of control cells was 82.0 ± 4.0 μg (mean of three cultures ± SD) at the beginning of the experiment, and 80.6 ± 5.0 μg (three cultures) after 144 h. Protein values were not decreased by treatment with cytokines for 72 h (IFN-γ/TNF-α, 82.0 ± 5.3 μg; IFN-γ/IL-1, 77.3 ± 1.1 μg; IFN-γ/LPS, 78.0 ± 2.0

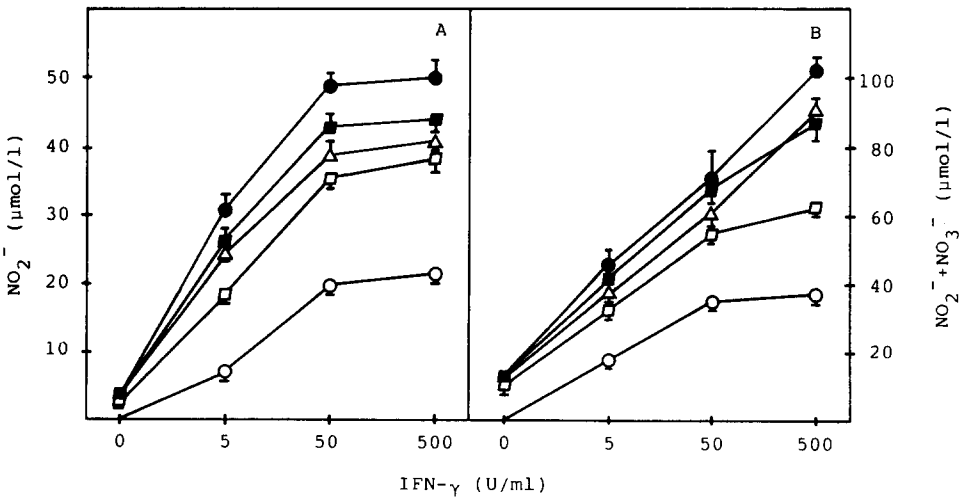

Figure 1. Formation of NO_2^- (*A*) and NO_2^- + NO_3^- (*B*) by murine fibroblasts as a function of IFN-γ concentration and enhancement of the IFN-γ effect by TNF-α, IL-1 and LPS. Confluent monolayers were treated with various doses of IFN-γ either alone (○) or in combination with 500 U/ml of TNF-α (●), 10 U/ml (□), or 100 U/ml (■) of IL-1, or 100 ng/ml of LPS (△) for 72 h. The results shown are means ± SD of triplicate cultures of one of three similar experiments in micromoles per liter. NO_3^- was enzymatically reduced to NO_2^-, which was detected by the Griess-reaction (see Materials and Methods).

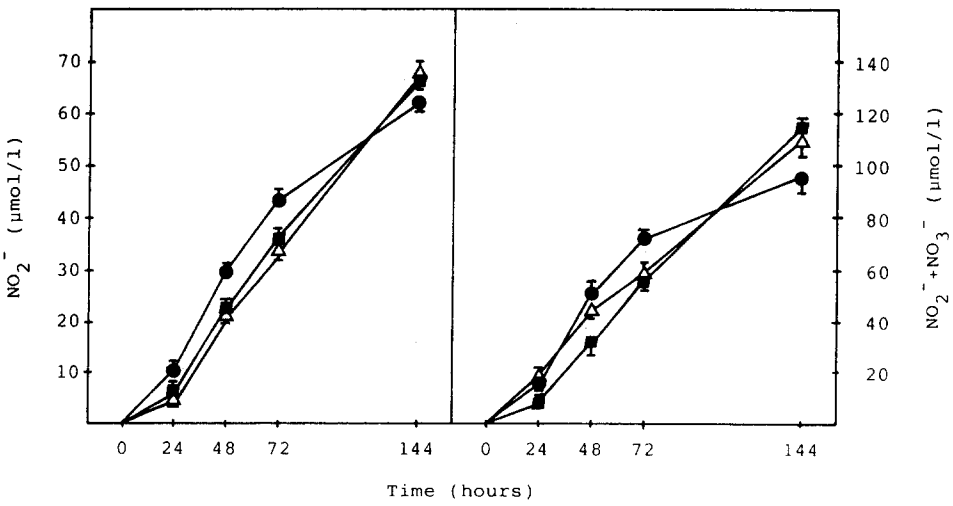

Figure 2. Time course of NO_2^- (*left*) and NO_2^- + NO_3^- (*right*) induction by murine fibroblasts treated with cytokine combinations. Confluent monolayers were stimulated with 50 U/ml of IFN-γ in combination with either 500 U/ml of TNF-α (●), 100 U/ml of IL-1 (■), or 10 µg/ml of LPS (△). Supernatants were harvested at indicated time points, and NO_2^- and NO_2^- + NO_3^- were determined as described in Materials and Methods, using the Griess reagent. Values are means of triplicate cultures ± SD from one of two similar experiments.

µg [three cultures]) as compared with controls. After 144 h, an ~20% reduction of protein content could be observed in cytokine-treated cells (IFN-γ/TNF-α, 62.6 ± 2.3 µg; IFN-γ/IL-1, 63.3 ± 1.1 µg; IFN-γ/LPS, 62.6 ± 3.0 µg [three cultures]). Again, NO_2^- was formed to 59 ± 11% (mean of 48 cultures ± SD) of NO_2^- + NO_3^- (Fig. 2).

Dependence of the Formation of NO_2^- and NO_3^- on L-arginine. When fibroblasts were stimulated with 5 U/ml of IFN-γ plus 500 U/ml of TNF-α in culture medium without L-arginine (see Materials and Methods), formation of NO_2^- was reduced to 1.8 ± 0.8 µmol/liter. In the presence of L-arginine (2.4 mM) 38.5 ± 4.0 µmol/liter (mean of six cultures ± SD) of NO_2^- was formed. NO_2^- + NO_3^- was reduced from 59.3 ± 5.1 µmol/liter in the presence of L-arginine to 5.5 ± 2.7 µmol/liter when stimulated without L-arginine (mean of six cultures ± SD, $p < 0.0001$, student's *t* test).

The L-arginine analogue NMA inhibited cytokine-induced formation of NO_2^- and NO_2^- + NO_3^- in a dose-dependent way (Fig. 3). Already, 25 µM of NMA was sufficient to decrease the amount of NO_2^- significantly from 38.4 ± 1.1 µmol/liter to 28.2 ± 0.7 µmol/liter (mean of three cultures ± SD, $p < 0.001$). Production of NO_2^- + NO_3^- was impaired in parallel. About 60 µM NMA was necessary for a 50% inhibition of nitrogen oxide formation (Fig. 3).

Effect of Modulation of Pteridine Synthesis on Formation of NO_2^- and NO_2^- + NO_3^-. Data given in Table 1 demonstrate that formation of NO_2^- upon treatment with IFN-γ alone or in combination with TNF-α, IL-1, or LPS was significantly decreased by treatment of cells with DAHP. This compound inhibits GTP-cyclohydrolase I, the key enzyme of tetrahydrobiopterin synthesis (22). The effect of IFN-γ on NO_2^- formation was thereby decreased to 22% ($p < 0.0001$), the effect of combined stimuli was reduced to ~30–50% ($p < 0.001$ for IFN-γ/IL-1 and IFN-γ/LPS; $p < 0.0001$ for IFN-γ/TNF-α). Addition of 100 µM sepiapterin, which provides tetrahydrobiopterin via a salvage pathway (2), reversed the effect of DAHP. Further, when given without DAHP, sepiapterin enhanced the effect of cytokine combinations on NO_2^- formation by 20–40%. In the case of IFN-γ alone, no such stimulation was observed (Table 1). Unconjugated pteridines not yielding tetrahydrobiopterin (2), such as pterin 6-carboxylic acid and isoxanthopterin, did not influence cytokine-induced NO_2^- formation or the effects of DAHP (Table 1).

Table 2 relates these findings to intracellular biopterin concentrations. Treatment of fibroblasts with IFN-γ/TNF-α resulted in an about threefold increase of intracellular tetrahydrobiopterin levels. Sepiapterin was converted into tetrahydrobiopterin in untreated control cells as well as in cells treated with IFN-γ/TNF-α. This conversion occurs due to sepiapterin reductase and further reduction of 7,8-dihydro-

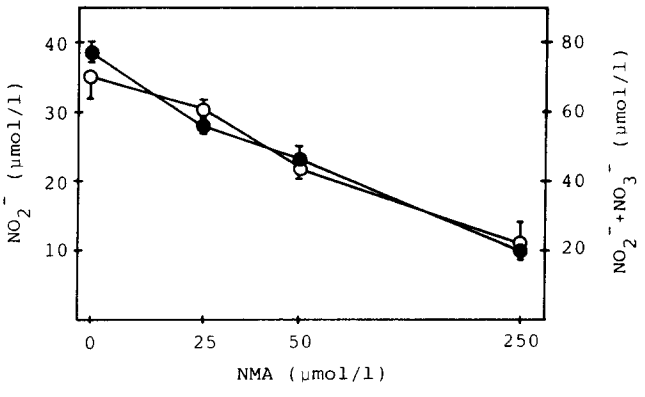

Figure 3. Effect of NMA on NO_2^- (○) and NO_2^- + NO_3^- (●) production in cytokine-treated murine fibroblasts. Confluent monolayers were pretreated with different concentrations of NMA for 24 h and then stimulated by 50 U/ml of IFN-γ plus 500 U/ml of TNF-α. After 72 h, NO_2^- and NO_2^- + NO_3^- were detected in supernatants by the Griess reaction (see Materials and Methods). One of two experiments showing the mean of triplicate cultures ± SD is presented here.

Table 1. *Influence of DAHP, Sepiapterin (SP), Pterin 6-carboxylic Acid (PCA), and Isoxanthopterin (IX) on Cytokine-induced Formation of NO_2^-*

Additive	NO_2^-			
	500 U/ml IFN-γ	50 U/ml IFN-γ + 500 U/ml TNF-α	50 U/ml IFN-γ + 500 U/ml IL-1	50 U/ml IFN-γ + 10 μg/ml LPS
		μmol/liter		
None	18.3 ± 0.6	38.8 ± 1.4	39.5 ± 2.6	34.2 ± 1.7
SP*	16.1 ± 1.0	57.9 ± 2.9	53.0 ± 6.1	40.8 ± 1.3
DAHP‡	4.1 ± 0.5	12.0 ± 1.6	18.2 ± 1.0	16.6 ± 1.0
SP + DAHP	17.0 ± 1.2	49.1 ± 0.5	38.2 ± 3.1	36.9 ± 1.8
PCA§	ND	34.0 ± 0.4	ND	ND
PCA + DAHP	ND	13.1 ± 1.3	ND	ND
IX‖	ND	34.9 ± 5.5	ND	ND
IX + DAHP	ND	12.0 ± 0.5	ND	ND

Confluent monolayers were treated with DAHP, pteridines, or both in the presence of IFN-γ alone or in combination with TNF-α, IL-1, or LPS for 72 h. NO_2^- was determined in supernatants as detailed in Materials and Methods. Values are means of triplicate cultures ± SD from one of three similar experiments. Control cells did not produce any detectable NO_2^- when treated with pteridines or DAHP.
* 100 μM sepiapterin.
‡ 5 mM DAHP.
§ 100 μM pterin 6-carboxylic acid.
‖ 100 μM isoxanthopterin.

Table 2. *Influence of DAHP, Methotrexate (MTX), and Sepiapterin (SP) on Synthesis of Biopterin and NO_2^- + NO_3^- in Cytokine-treated Murine Fibroblasts*

Treatment	Total intracellular biopterin	$NO_2^- + NO_3^-$ in supernatants
	pmol/mg	*μmol/liter*
None	18.7 ± 1.0 (96.1 ± 5.7)	ND
SP*	5,358.0 ± 413.4 (94.0 ± 0.5)	ND
DAHP‡ + SP	3,801.3 ± 149.6 (89.9 ± 0.1)	ND
MTX§	18.7 ± 1.6 (56.9 ± 4.5)	ND
IFN-γ/TNF-α‖	57.9 ± 3.0 (89.9 ± 2.1)	127.2 ± 11.6
IFN-γ/TNF-α + SP	1,661.5 ± 239.9 (95.5 ± 1.4)	197.8 ± 17.3
IFN-γ/TNF-α + DAHP	24.4 ± 4.3 (>98)	72.7 ± 8.1
IFN-γ/TNF-α + DAHP + SP	1,757.8 ± 541.2 (91.8 ± 4.0)	131.0 ± 13.3
IFN-γ/TNF-α + MTX	53.5 ± 1.0 (73.5 ± 3.0)	124.2 ± 15.2
IFN-γ/TNF-α + MTX + SP	153.4 ± 27.7 (18.4 ± 8.4)	70.0 ± 15.9
IFN-γ/TNF-α + DAHP + MTX	16.0 ± 5.5 (81.1 ± 0.9)	62.6 ± 14.0
IFN-γ/TNF-α + DAHP + MTX + SP	180.3 ± 2.7 (14.0 ± 11.9)	20.1 ± 5.6

Confluent monolayers, grown in 25-cm^2 culture flasks, were treated with various additives and cytokines for 72 h. Total intracellular biopterin was determined with HPLC after iodine oxidation at acidic pH. The amount of biopterin plus 7,8-dihydrobiopterin was assessed by iodine oxidation at alkaline pH. Values in parantheses give the percentage of tetrahydrobiopterin, which was calculated from the ratio of total biopterin to biopterin plus 7,8-dihydrobiopterin. Nitrogen oxides were determined in supernatants. For experimental details, see Materials and Methods. NO_2^- in supernatants was 47 ± 10% of NO_2^- + NO_3^- (mean of 36 cultures ± SD). Values are means of triplicate cultures ± SD from one of two experiments.
* 100 μM sepiapterin.
‡ 5 mM DAHP.
§ 10 μM methotrexate plus 40 μM thymine and 100 μM inosine.
‖ 50 U/ml IFN-γ + 500 U/ml TNF-α.

Table 3. *Influence of DAHP, Sepiapterin, and Arginine on Cell Viability of Murine Fibroblasts Treated with Cytokines*

Treatment	Relative cell viability after: 48 h	72 h	96 h	NO_2^- after 96 h
		%		*μmol/liter*
None	100 ± 9	100 ± 7	100 ± 13	3.5 ± 1.4
IFN-γ/TNF-α*	86 ± 8	73 ± 4	45 ± 6	57.4 ± 3.9
IFN-γ/TNF-α + SP‡	80 ± 6	75 ± 10	50 ± 6	83.0 ± 6.7
IFN-γ/TNF-α + DAHP§	91 ± 6	89 ± 6	70 ± 7	21.3 ± 3.2
IFN-γ/TNF-α + SP + DAHP	77 ± 5	65 ± 4	54 ± 6	69.1 ± 9.4
IFN-γ/TNF-α + NMA‖	81 ± 4	71 ± 3	78 ± 7	15.0 ± 2.3
IFN-γ/TNF-α + NMA + L-ARG¶	77 ± 12	68 ± 9	47 ± 2	65.9 ± 3.1
IFN-γ/TNF-α + NMA + D-ARG**	94 ± 6	95 ± 5	88 ± 4	9.3 ± 2.4
IFN-γ/TNF-α + L-ARG‡‡	70 ± 3	55 ± 2	22 ± 5	71.4 ± 5.4
IFN-γ/TNF-α + D-ARG§§	94 ± 6	94 ± 5	89 ± 3	8.8 ± 1.2
IFN-γ/TNF-α without L-ARG‖‖	86 ± 6	88 ± 5	89 ± 3	3.9 ± 1.2

Confluent monolayers of murine fibroblasts were grown in 96-well plates and treated with IFN-γ/TNF-α in absence or presence of DAHP, sepiapterin (SP), high doses of L- or D-arginine with or without NMA, and in L-arginine-deficient medium. At the indicated time points, cells were stained with methylene blue, and OD at 620 nm was determined after solubilizing the dye with 0.33 M HCl (see Materials and Methods). Values are expressed as percent of viable cells relative to untreated controls (= 100%) and give the mean of eight wells ± SD determined in one of three similar experiments. Controls after 96 h were 112 ± 7 for sepiapterin, 108 ± 7 for DAHP, 96 ± 7 for NMA, 94 ± 4 for NMA plus 10 mM L-arginine, 98 ± 7 for NMA plus 10 mM D-arginine, 102 ± 6 for 10 mM L-arginine, 96 ± 5 for 10 mM D-arginine, and 95 ± 7 for L-arginine-deficient medium. NO_2^- in the respective wells was determined using the Griess reaction.

* 50 U/ml IFN-γ + 500 U/ml TNF-α.
‡ + 100 μM sepiapterin.
§ + 5 mM DAHP.
‖ + 250 μM NMA.
¶ + 250 μM NMA + 10 mM L-arginine.
** + 250 μM NMA + 10 mM D-arginine.
‡‡ + 10 mM L-arginine.
§§ + 10 mM D-arginine.
‖‖ Stimulated in L-arginine-deficient medium.

biopterin into tetrahydrobiopterin by dihydrofolate reductase (EC 1.5.1.3) (2). DAHP reduced intracellular tetrahydrobiopterin levels by ~50%. Concurrent addition of DAHP and sepiapterin resulted in high intracellular tetrahydrobiopterin levels, restoring the inhibition of nitrogen oxide formation by DAHP. This reversal was inhibited by methotrexate, which interferes with formation of tetrahydrobiopterin from sepiapterin by inhibition of dihydrofolate reductase (2), thus leading to the accumulation of dihydrobiopterin. Intracellular accumulation of dihydrobiopterin even had an inhibitory effect on synthesis of NO_2^- and NO_2^- + NO_3^-, as can be seen from comparison of the effects of methotrexate alone or together with sepiapterin in IFN-γ/TNF-α–stimulated cells ($p < 0.01$) (Table 2). This may occur due to inhibition of GTP-cyclohydrolase I by dihydrobiopterin (23). As a consequence, the combination of DAHP, methotrexate, and sepiapterin had the highest inhibiting potency.

Cell Viability. Cytokine-induced formation of nitrogen oxides leads to inhibition of aconitase (EC 4.2.1.3) in tumor cells (24) as well as in activated macrophages (15, 25). Further, it was reported previously, that IFN-γ alone or in combination with IL-1, TNF-α, or LPS is cytotoxic for murine fibroblasts (26, 27). We therefore studied the effect of IFN-γ/TNF-α on cell viability in the absence or presence of DAHP or sepiapterin (Table 3). After incubation for 96 h with IFN-γ/TNF-α, a decrease of cell viability to ~50% became apparent. The same was observed with IFN-γ in combination with IL-1 or LPS, and was less pronounced with IFN-γ alone (not shown). Table 3 shows that the cytotoxic effect of IFN-γ/TNF-α could be partially reversed by simultaneous treatment with DAHP ($p < 0.01$). Concurrent treatment with sepiapterin restored the cytotoxic effect of IFN-γ/TNF-α, thus paralleling the effects on NO_2^- formation. On control cells, neither DAHP nor sepiapterin could influence cell viability (Table 3). The cytotoxic effect of IFN-γ/TNF-α was also reduced by treatment with NMA or by withdrawal of L-arginine. This effect of NMA was reversed by addition of L-arginine but not by D-arginine (Table 3). Further, addition

of 10 mM L-arginine but not of D-arginine to cytokine-treated cells in absence of NMA resulted in the highest cytotoxic effect (Table 3) ($p < 0.0001$ as compared with NMA plus 10 mM L-arginine). Cell viability was significantly correlated to NO_2^- levels in supernatants of the respective wells (Table 3), as determined by linear regression analysis ($R = -0.78$, $p < 0.000001$; 144 samples) and Spearman Rank correlation ($R = -0.84$, $p < 0.000001$; 144 samples).

Discussion

In this report, we demonstrate that murine fibroblasts are capable to produce NO_2^- and NO_3^- from L-arginine upon stimulation with IFN-γ. This effect of IFN-γ is enhanced up to threefold by costimulation with TNF-α, IL-1, or LPS. So far, cytokine-induced formation of nitrogen oxides has been observed in murine macrophages, murine macrophage cell lines (12–15, 28), rat Kupffer cells, rat hepatocytes (29, 30), and murine endothelial cells (31). As in murine macrophages, NO_2^- is formed in fibroblasts to ~50% of the totally detectable NO_2^- + NO_3^- (13). Further, fibroblasts produce NO_2^- and NO_3^- to an extent comparable with that found for macrophages (13), macrophage cell lines (28), and endothelial cells (31). In murine macrophages, the effect of IFN-γ on formation of nitrogen oxides was enhanced by TNF-α, TNF-β, or LPS but, in contrast to our results with fibroblasts, not by IL-1 (14, 15). The significance of this difference is unclear.

NO_2^- and NO_3^- originate from the oxidation of L-arginine to citrulline. This reaction can be inhibited by the L-arginine analogue NMA (32, 33). The unstable intermediate formed initially is nitric oxide, which is converted to NO_2^- and NO_3^- in the presence of oxygen (34). Nitric oxide has been identified as a cytotoxic effector molecule of activated murine macrophages (35, 36).

Recently, it was shown in preparations from cytosol of activated macrophages that oxidation of L-arginine requires tetrahydrobiopterin (8, 9). Tetrahydrobiopterin was suggested to take part in the initial step of the reaction, resulting in *N*-hydroxylation of L-arginine (8, 9). It was further shown that murine macrophages contain tetrahydrobiopterin (8, 9). We show here for murine fibroblasts that cytokine-induced formation of NO_2^- and NO_3^- is paralleled by increased intracellular levels of tetrahydrobiopterin. By inhibiting tetrahydrobiopterin de novo synthesis, using the GTP-cyclohydrolase I inhibitor DAHP (22), we could demonstrate that the amount of intracellular tetrahydrobiopterin is limiting the formation of NO_2^- and NO_3^-. Resupplementing the cofactor by addition of sepiapterin, which is converted into tetrahydrobiopterin in the cells via a salvage pathway (2), fully restores the formation of NO_2^- and NO_3^-. Methotrexate blocks this restoring effect of sepiapterin by inhibiting dihydrofolate reductase, which converts dihydrobiopterin formed from sepiapterin to the tetrahydro-derivative (2), thus underlining that only the tetrahydroderivative can serve as an active cofactor. While inhibition of tetrahydrobiopterin concentrations by DAHP significantly suppresses nitrogen oxide formation, a comparatively high amount of sepiapterin, and thus a high concentration of tetrahydrobiopterin, is necessary for restoration of nitrogen oxide formation. A possible explanation for this phenomenon could be that tetrahydrobiopterin generation from exogenously supplied sepiapterin may take place in a compartment different from the site of arginine hydroxylation, whereas endogenous tetrahydrobiopterin synthesis may supply tetrahydrobiopterin directly to the hydroxylation site.

Other unconjugated pteridines that cannot be converted to tetrahydrobiopterin due to the lack of the 1',2'-dihydroxypropyl side chain at position 6 of the pterin ring (2) could not influence formation of nitrogen oxides and the effect of DAHP. Sepiapterin also enhanced the effect of IFN-γ in combination with TNF-α, IL-1, or LPS, but not with IFN-γ alone. Thus, it seems that in presence of dual activation stimuli, the reaction forming NO_2^- is not saturated with tetrahydrobiopterin, although its synthesis is enhanced in parallel.

Taken together, the results obtained with DAHP, sepiapterin, and methotrexate give evidence that not only in cytosol preparations (8, 9), but also in intact cells, tetrahydrobiopterin is essential for the production of nitrogen oxides.

The cytotoxic effect of IFN-γ in combination with TNF-α, IL-1, or LPS on murine fibroblasts can be attenuated by reducing formation of nitrogen oxides via inhibition of L-arginine hydroxylation with NMA, via withdrawal of L-arginine, or via inhibition of tetrahydrobiopterin synthesis with DAHP. Addition of L-arginine and sepiapterin, respectively, restores the cytotoxic potential of cytokine treatment. These data indicate that formation of nitrogen oxides takes part in the previously reported cytotoxic action of IFN-γ with TNF-α, IL-1, or LPS (26, 27) on murine fibroblasts. Thus, it is not only possible to influence formation of NO_2^- and NO_3^- by modulation of pteridine synthesis, but also, the biological effect of nitrogen oxides on fibroblasts.

The presented data suggest that in murine fibroblasts a major goal for cytokine-induced pteridine synthesis is to provide cells with an essential cofactor for the formation of nitrogen oxides from L-arginine. Nitric oxide, the initial product of the oxidation of L-arginine, exhibits a multitude of biological actions (37, 38). It remains to be seen whether also in human cells cytokine-induced pteridine synthesis (1, 3–7) is related to oxidation of L-arginine and formation of nitrogen oxides.

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