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AVAILABILITY OF TETRAHYDROBIOPTERIN IS NOT A FACTOR IN THE INABILITY TO DETECT NITRIC OXIDE PRODUCTION BY HUMAN MACROPHAGES

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SUMMARY: Human macrophages, in contrast to murine macrophages, do not produce nitric oxide after stimulation with cytokines. This failure has been attributed to the known lack of production by human macrophages of tetrahydrobiopterin, an essential cofactor for nitric oxide synthase. Increasing intracellular levels of tetrahydrobiopterin in cytokine-stimulated murine cells results in an increase in nitrite production. However, this treatment does not result in any detectable accumulation of nitrite by stimulated human monocyte-derived macrophages. Thus, the inability of these cells to produce nitric oxide appears to be unrelated to a lack of tetrahydrobiopterin and suggests that proper *in vitro* conditions may not yet have been discovered that permit nitric oxide synthesis by activated human macrophages.

There are two distinct types of enzymes which produce nitric oxide from L-arginine (1). Endothelial cells, neurons, smooth muscle cells, and platelets contain the constitutive, calcium-dependent type of nitric oxide synthase which produces small amounts of nitric oxide, presumably to use as an inter or intracellular signaling molecule in the regulation of numerous physiological processes (2). In contrast to this low output system, the cytokine-inducible, high output type of nitric oxide synthase which is calcium independent has been found in murine macrophages, hepatocytes, and vascular smooth muscle cells (3). Reactive nitrogen intermediates (RNI), nitric oxide, nitrite, or nitrate, produced in these cells during the oxidation of arginine to citrulline are thought to play a role in cytostatic and cytolytic activity of macrophages towards invading organisms.

Although numerous studies have been carried out on inducible, L-arginine-dependent, nitric oxide synthesis by rodent macrophages, with the notable exception of two reports (4, 5), others have been unable to detect cytokine-stimulated production of RNI by human macrophages

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ABBREVIATIONS: NO, nitric oxide; RNI, reactive nitrogen intermediates; IFN-γ, interferon-gamma; TNF-α, tumor necrosis factor-alpha; BH_4, tetrahydrobiopterin; SP, sepiapterin (6-lactoyl-7,8-dihydropterin); DAHP, 2,4-diamino-6-hydroxypyrimidine; DMEM, Dulbecco's minimum essential medium.

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(6, 7, 8, 9). Recently, Hibbs et al have presented evidence for high output nitric oxide synthesis *in vivo* in humans treated with high doses of interleukin 2 for anti-cancer therapy (10). Cameron et al (7) have suggested that the failure to demonstrate nitric oxide production by cytokine-stimulated human macrophages may be a consequence of the known inability of these cells to synthesize tetrahydrobiopterin which is a required cofactor in the nitric oxide synthase reaction. They further proposed that activated macrophages *in vivo* could acquire tetrahydrobiopterin from other cells such as lymphocytes (7). In this study, we investigated the effect of supplementing human macrophages with tetrahydrobiopterin on nitric oxide production and found that a lack of synthesis of this cofactor is not responsible for the failure to detect cytokine-inducible nitrite production.

MATERIALS AND METHODS

Materials. Recombinant mouse and human IFN-γ and human TNF-α were from R&D Systems (Minneapolis, MN). DAHP and LPS were obtained from Sigma Chemical Co. (St. Louis, MO,). SP was from B. Schircks Laboratory (Jona, Switzerland). All other reagents were of highest purity available and obtained from various local sources.

Cells and cell culture. Normal human macrophages, purified by elutriation and cultured for 7-10 d in DMEM containing 20% fetal bovine serum and 10% human AB serum were purchased from ABI (Columbia, MD). In some experiments, mononuclear cells were isolated from buffy coats and purified by ficoll centrifugation (Sigma Chemical Co). Monocytes were selected by adherence to plastic tissue culture flasks, and cultured as described above for various times. Normal human fibroblasts were obtained from Dr. George Hoganson, University of Chicago Medical Center and were cultured in DMEM containing 10% fetal bovine serum. Thioglycollate-elicited peritoneal macrophages were isolated from adult rats 48 h after intraperitoneal injection of 5 ml of thioglycollate broth and cultured in DMEM containing 10% fetal bovine serum. Other cell lines were from the American Type Culture Collection and were cultured as suggested by the supplier. Treatments with cytokines, pterins, and inhibitors were as described in Table legends. Macrophages and THP-1 cells were harvested by scraping. Other cells were usually harvested with trypsin and all cells were extracted by sonication in 50 mM Tris-HCl (pH 7.4)/1 mM dithiothreitol/0.1 mM EDTA.

Pterin and nitrite determinations. Pterins were measured by HPLC with fluorescence detection after oxidation of reduced forms as previously described (11). The fraction of total biopterin present as the tetrahydro form was determined by a differential oxidation method (12) in selected samples from each experiment and was always >95% of the total. Nitric oxide production was evaluated by measuring the rate of accumulation of nitrite in cell media (13). Nitrite was measured by the Griess reaction modified for use in a microplate reader (6). Nitrite standards were prepared in the same media being analyzed.

RESULTS AND DISCUSSION

Tayeh and Marletta discovered that the enzymatic reaction which converted L-arginine to nitric oxide and L-citrulline was tetrahydrobiopterin-dependent while purifying nitric oxide synthase from the cytokine-stimulated murine macrophage cell line, RAW 264.7 (14). Subsequently, an absolute requirement of *de novo* BH_4 biosynthesis for nitric oxide production has been demonstrated in murine fibroblasts (15), bovine aortic endothelial cells (16), murine brain endothelial cells (17), human umbilical vein endothelial cells (18), vascular smooth muscle

cells (19), and RAW 264 cells (11). Conspicuously, only few reports of high output nitric oxide production by cytokine-treated human cells have appeared. It has been reported that Mycobacterium-infected monocyte-derived macrophages produce nitric oxide after treatment with IFN-γ (4). In addition, Hunt and Goldin found that monocytes isolated from a control, non-alcoholic group produced high levels of nitrite after stimulation with endotoxin while those from patients with alcoholic liver disease constitutively produced a high level of nitric oxide which was not further increased by endotoxin (5). Recently, it has also been demonstrated that human hepatocytes (20), myocardium (21), and megakaryocytes (22) have cytokine-inducible NOS activity. Further work is needed to confirm these studies. In fact, several groups have reported that human macrophages can **not** produce RNI from L-arginine (6, 7 ,8 ,9) which suggests that this process may be either a species-specific response to invading organisms or that nitric oxide synthase may be inactive *in vitro* since human macrophages produce little (if any) tetrahydrobiopterin (23).

Recently, we found that cytokine-stimulated nitric oxide production by murine macrophages was relatively insensitive to removal of most of the BH_4 (>95%) from the cells because of the high affinity of NOS for BH_4 (11). Thus, it seems plausible that human macrophages could acquire sufficient BH_4 from extracellular sources to support nitric oxide synthesis *in vivo*. We carried out preliminary experiments to determine optimum conditions for cytokine-stimulated pterin synthesis by human macrophages. In agreement with other studies (24), we found a dose-dependent stimulation by IFN-γ, as determined by neopterin production (Table I). However, monocyte-derived macrophages did not produce detectable increases of

TABLE I. Lack of effect of cytokines and pterin supplementation on production of nitric oxide by human monocyte-derived macrophages

Cells	Neopterin (pmol/10^6 cells)	Biopterin (pmol/10^6 cells)	Nitrite (μM)
A. Monocyte/Macrophages, 48 h			
control	66.0 ± 6.3	ND	2.33 ± 0.07
IFN-γ 10 U	379 ± 62.8	ND	2.47 ± 0.18
IFN-γ 100 U	507 ± 26.9	ND	2.40 ± 0.12
IFN-γ 1000 U	501 ± 139	ND	1.93 ± 0.07
B. Monocyte/Macrophages, 24 h			
IFN-γ + TNF-α + SP	15.7 ± 1.6	8358 ± 1331	0.7 ± 0.2
C. THP-1 Monocytic, 24 h			
control	ND	ND	0.38 ± 0.15
IFN-γ	186 ± 2.2	2.71 ± 0.11	0.78 ± 0.15
IFN-γ + DAHP	4.81 ± 0.73	ND	0.46 ± 0.21
IFN-γ + SP	6.33 ± 1.07	11872 ± 1171	ND

Macrophage cultures, prepared as described in Materials and Methods, were treated with the indicated agents (IFN-γ, 250 U/ml; TNF-α, 100 U/ml; DAHP, 5 mM; SP, 100 μM) in DMEM containing 10% fetal bovine serum. Nitrite in the media and intracellular pterins were measured as described in Materials and Methods. The results are expressed as means ± SD. ND, none detected (<0.2 pmol/10^6 cells for pterins and <0.25 μM for nitrite).

either BH_4 or of nitrite after treatment with IFN-γ. Similar lack of response was found in experiments on macrophages isolated from 10 other normal controls. Furthermore, various combinations of IFN-γ, TNF-α, LPS, and GM-CSF for periods of up to one week also did not result in detectable nitrite accumulation (data not shown).

However, it should be noted that stimulated human macrophages produce little (if any) BH_4, which is absolutely required for nitric oxide synthase activity, since they have limiting levels of the second enzyme of the de novo BH_4 biosynthesis pathway, 6-pyruvoyl tetrahydropterin synthase (23, 24). In order to investigate the possibility that nitric oxide synthesis *in vitro* could be limited by the availability of BH_4, we studied the effect of adding BH_4 to IFN-γ-treated human monocyte/macrophages on the production of nitrite. Because BH_4 is unstable under usual tissue culture conditions, cells were treated with sepiapterin which is converted intracellularly to BH_4 by sequential reductions catalyzed by sepiapterin and dihydrofolate reductases, respectively (25). When macrophages were treated with sepiapterin and cytokines, there was again no detectable nitrite production although this treatment resulted in intracellular BH_4 levels 15-fold higher than the increased neopterin levels found in macrophages treated with cytokines alone.

The human monocytic THP-1 cell line has somewhat different properties than primary monocyte/macrophages. These cells apparently produce low levels of nitrites which do not increase after treatment with cytokines (Table I). As in the case of primary monocyte/macrophages, they only synthesize low amounts of pterins in the absence of cytokines. Treatment of THP-1 cells with IFN-γ stimulated the production of a large amount of neopterin with the concomitant formation of lower, but significant concentrations of BH_4 (Table 1). However, inhibition of pterin synthesis with DAHP had no effect on the small amount of nitrite constitutively produced by these cells. Furthermore, increasing the intracellular concentration of BH_4 by incubation with sepiapterin, also did not result in any increase in nitrite accumulation.

In addition, human U373 glioblastoma cells which produce BH_4 in response to IFN-γ, do not produce nitrite even when supplemented with exogenous BH_4 (Table II). Stimulation of murine fibroblasts with IFN-γ has been shown to result in the induction of both BH_4 synthesis and nitric oxide production (15). In contrast, treatment of human fibroblasts with cytokines only stimulates production of pterins with no detectable accompanying production of nitrite (Table II).

We have previously shown that nitric oxide synthesis by cytokine-stimulated, BH_4-depleted, murine macrophage RAW 264 cells can be restored by addition of BH_4 (11). To demonstrate that high levels of BH_4 do not interfere with nitric oxide production, we treated RAW 264 cells with sepiapterin for 24 h to increase BH_4 levels in the presence of IFN–γ and LPS (Table III). There was a 50% increase in nitrite accumulation when BH_4 levels were increased more than 40 fold, suggesting that nitric oxide synthase in these cells is normally not completely saturated with BH_4 and also that neither sepiapterin nor excess BH_4 have deleterious effects on nitric oxide synthesis. Furthermore, this stimulatory effect was not limited to only a macrophage cell line since we also found similar increases in nitric oxide production by sepiapterin-supplemented, primary peritoneal macrophage cultures which were activated with IFN–γ and LPS (Table III).

TABLE II. Lack of nitrite production by cytokine-treated human glioblastoma cells or fibroblasts is unrelated to intracellular pterin levels

Cells	Treatment	Neopterin (pmol/10^6 cells)	Biopterin (pmol/10^6 cells)	Nitrite (μM)
U373 Glioblastoma	none	ND	ND	ND
	IFN-γ	ND	40.7 ± 3.7	ND
	IFN-γ + DAHP	ND	ND	ND
	IFN-γ + SP	ND	15010 ± 2801	ND
Skin Fibroblasts	none	ND	ND	ND
	IFN-γ + TNF-α	34.4 ± 8.8	1944 ± 140	ND

Confluent cultures were treated for 24 h with the indicated agents (IFN-γ, 250 U/ml; TNF-α, 100 U/ml; DAHP, 5 mM; SP, 100 μM) in DMEM containing 10% fetal bovine serum. Nitrite in the media and intracellular pterins were measured as described in Materials and Methods. The results are expressed as means ± SD of triplicate determinations. ND, none detected (<0.2 pmol/10^6 cells for pterins and <0.25 μM for nitrite).

In summary, it appears that a deficiency of BH_4 in human macrophages is not a factor in the failure to detect production of RNI. Experiments with other types of cells from humans and other species, including murine macrophages, have shown that BH_4 supplementation can restore nitric oxide synthase activity to cells depleted of BH_4. Thus, it seems unlikely that the large increases in intracellular BH_4 concentrations in our experiments with human macrophages would not have been effective in stimulating nitric oxide production, if indeed these cells contained BH_4-deficient nitric oxide synthase activity. We must conclude that acquisition of BH_4 by human macrophages is not a limiting factor in the production of RNI and that the failure to detect formation of nitric oxide *in vitro* means that either human macrophages do not possess a high

TABLE III. Effect of pterin supplementation on nitrite production by cytokine-activated murine macrophages (RAW 264) and peritoneal macrophages

Cells		Biopterin (pmol/10^6 cells)	Nitrite (μM)
RAW 264	none	14.2 ± 0.42	0.28 ± 0.2
	IFN-γ + LPS	9.73 ± 0.44	61.8 ± 0.87
	IFN-γ + LPS + DAHP	2.57 ± 0.38	48.9 ± 1.81*
	IFN-γ + LPS + SP	201 ± 8.17	77.5 ± 2.01*
Macrophages	none	0.53 ± 0.28	ND
	IFN-γ + LPS	10.1 ± 0.84	61.3 ± 3.67
	IFN-γ + LPS + SP	45.9 ± 3.38	73.8 ± 4.74*

RAW cells were treated for 24 h with IFN-γ (25 U/ml) and LPS (1.0 ng/ml) in the absence or presence of DAHP (5 mM) or SP (50 μM). Rat peritoneal macrophages were also treated for 24 h with IFN-γ (10 U/ml) and LPS (1.0 ng/ml) in the absence or presence of SP (50 μM). Cells were harvested and total cellular content of BH_4 and nitrite concentration in the media were measured as described in Materials and Methods. Since NO is cytotoxic, cell numbers are lower 24 h after cytokine treatment than in control cultures. Correction of the results for this decrease, which is more important for intracellular biopterin levels, was not made since SP had no additional significant effects on cell numbers. The results are expressed as means ± SD of triplicate determinations. * $P \leq 0.01$ compared to cells treated with IFN-γ and LPS. ND, none detected (<0.25 μM for nitrite).

output nitric oxide system and increased RNI detected *in vivo* (10) arises from other cell types or that experimental conditions have not yet been found that mimic the appropriate repertoire of cytokines, growth factors, hormones, cofactors, etc, which lead to high output nitric oxide synthesis *in vivo*.

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