Nitrite Generation in Interleukin-4–Treated Human Macrophage Cultures Does Not Involve the Nitric Oxide Synthase Pathway

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The search continues for high-output nitric oxide biosynthesis in human macrophages analogous to murine phagocytes. Recently, generation of nitrite in culture supernatants of human macrophages exposed to interferon-γ and interleukin-4 (IFN-γ/IL-4) was reported. The present study reproduces these findings and shows that L-arginine is not consumed and L-citrulline is not produced during this process. Furthermore, the biosynthesis of the obligatory cofactor tetrahydrobiopterin is not coinduced. These biochemical data provide support against a nitric oxide synthase contribution to nitrite accumulation. Nitrite was generated from nitrate salts even in cell-free media. Nitric oxide synthase activity but not nitrate reduction depended on molecular oxygen. Nitrite accumulation in experiments with IFN-γ/IL-4 in human monocytes appears to be an in vitro artifact produced by nitrate-reducing activities contained in cytokine preparations.

In 1985, the secretion of nitrite by lipopolysaccharide (LPS)-activated and interferon-γ (IFN-γ)–activated murine macrophages was described [1] and ultimately attributed to the enzymatic activity of an inducible nitric oxide synthase (NOS). An analogous high-output nitrite-producing system has not been found in human macrophages [2–4]; nevertheless, some laboratories have reported nitrite accumulation in human phagocyte cultures and suggested that it originates from a functional NOS analogous to the murine enzyme [5, 6]. Those findings were not confirmed by other laboratories [7, 8].

In 1994 and 1995, new data indicated that human monocytes isolated from certain individuals generate nitrite in micromolar amounts in long-term cultures when exposed to IFN-γ combined with interleukin-4 (IFN-γ/IL-4) [9–15]. Those studies depended on the measurement of nitrite in culture supernatants without more specific tests for NOS activity, such as the assessment of substrate consumption (L-arginine) and formation of the coproduct (L-citrulline) of NOS. Furthermore, they did not test for tetrahydrobiopterin biosynthesis, the obligate cofactor of NOS activity that is coinduced upon inflammatory stimulation in murine macrophages and human hepatocytes [16, 17] but not in human macrophages [3, 18].

The present study was designed to evaluate nitric oxide biosynthesis by human macrophages exposed to IFN-γ/IL-4, examining the specific biochemical parameters of the NOS pathway (i.e., L-arginine consumption, L-citrulline production, and tetrahydrobiopterin biosynthesis). Nitrite formation in supernatants of human macrophage cultures was compared with nitrite generation in cell-free medium incubated under identical experimental conditions. Additional experiments using incubation in anaerobic chambers were done to further study the source of nitrite in culture systems that do or do not display high output NOS activity.

Materials and Methods

Isolation of mononuclear cells and cell culture. Human mononuclear cells were isolated from healthy donors, including 2 white males (donors 1 and 2), 1 Chinese female (donor 3), and 1 Asian Indian male (donor 4), as described previously [3]. In brief, cells were separated from heparinized blood by a Ficoll (Pharmacia, Uppsala, Sweden) gradient and seeded ($0.5 \times 10^5/cm^2$ culture surface) on 100-mm plastic tissue culture dishes (Falcon Plastics, Oxnard, CA) in 10 mL of Iscove's modified Dulbecco's medium (IMDM; GIBCO, Basel, Switzerland) supplemented with 25% pooled human serum and 50 μg/mL gentamicin (Schering, Kenilworth, NJ). The plates were incubated for 2 h at 37°C in a 5% CO_2 atmosphere (Steri-Cult; Forma Scientific, Marietta, OH), and then nonadherent cells were removed. The adherent cells were incubated for the times indicated in IMDM supplemented as above and further supplemented with 500 U/mL recombinant human IFN-γ (Biogen, Geneva), 20 ng/mL recombinant human IL-4 (Schering), 1 m*M* N^G-monomethyl-L-arginine (L-NMMA; Calbiochem, La Jolla, CA), 1 m*M* *N*-ω-nitro-L-arginine (L-NNA; Sigma), or 5 m*M* L-arginine (tissue culture grade; Sigma) as indicated.

Rat aortic smooth muscle cells (SMCs) [19] were cultured in IMDM with 10% fetal calf serum (FCS) and 50 μg/mL gentamicin in 100-mm tissue culture dishes (Falcon). At confluence, they were stimulated with 10 μg/mL LPS (Boivin extraction 026:B6; Difco, Detroit) and 100 U/mL recombinant rat IFN-γ (Innogenetics, Zwijndrecht, Belgium). RPMI 1640, IMDM, Medium 199 (M-

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199), and MEM were all from GIBCO, and FCS (specified low IgG and low endotoxin) was from PAA (Linz, Austria). Supernatants were collected after the indicated incubation times for determination of nitrite, amino acids, and pterins. All media and supplements were free of azide and endotoxin (<0.1 ng/mL) as specified by the manufacturer and confirmed by negative limulus lysate assay. Cells were >95% viable monocytes or SMCs, as assessed by trypan blue dye exclusion and Giemsa staining. Microbial contamination of cultures was excluded by negative bacterial growth in aliquots of complete media and culture supernatants on blood and chocolate agar plates.

Anaerobic culture system. For cultures grown under anaerobic conditions, cells were incubated for the indicated times at 37°C in a Difco anaerobic system according to the manufacturer's instructions; within 1 h, the atmosphere was <0.02% O_2 and 4%–10% CO_2. Anaerobic conditions were verified using the system's indicator strips and a methylene blue redox indicator assuring a redox potential of less than −230 mV.

Quantitation of nitrite. Nitrite was measured in culture media at the indicated times using equal volumes of sample and Griess reagent (0.05% *N*-1-naphthylethylenediamine dihydrochloride and 0.5% sulfanilamide in 2.5% phosphoric acid) in 96-well microplates. A Micro-ELISA Autoreader (Dynatech, Kloten, Switzerland) was used as described previously [3, 16]; techniques were similar to those in studies of long-term cultures of human macrophages [9–15]. The detection limit was 10 pmol/microwell. Nitrite concentrations were calculated from sodium nitrite standards diluted in the respective appropriate medium.

Nitrate reduction. For reduction of nitrate salts to nitrite, media or sera were incubated with 120 mU of nitrate reductase (*Aspergillus niger;* Boehringer Mannheim, Mannheim, Germany) and 100 μM NADPH (Sigma) for 2 h at room temperature. For reduction of RPMI 1640, 1 m*M* NADPH was used. Nitrite was quantitatively detected by the Griess reaction as outlined above. Nitrate standards identically reduced as samples were used for calculation.

Amino acid analysis. Contents of amino acids in culture media were quantitated by a routine procedure using an LC 5001 Autoanalyzer equipped with BTC 2710 cation exchange resin (Biotronik, Maintal, Germany) as described [3]. The detection limit for L-arginine, L-ornithine, L-citrulline, and the other amino acids was 2–5 μM.

Measurement of intracellular pterins. Pterins in lysates of cultured human macrophages were measured after acidic oxidation by high-performance liquid chromatography (HPLC) using a reverse-phase analytical column (Spherisorb ODS1; Stagroma, Wallisellen, Switzerland) and fluorimetric detection by a fluorimeter (S 1000; Hitachi Scientific Instruments, Mountain View, CA) as described [16]. The detection limit was <0.1 pmol/10^6 cells.

Statistical analysis. Values are given as mean ± SE. Small differences between mean values were evaluated for statistical significance by Student's *t* test.

Results

Human blood-derived macrophages from 4 different donors were cultured for up to 12 days in the presence of IFN-γ/IL-4 or medium alone. No measurable changes in nitrite concentration were observed in any cultures during the first 7 days. From day 9 on, however, the nitrite concentration rose significantly in supernatants from 2 subjects' cells cultured with IFN-γ/IL-4; nitrite generation was less pronounced in cell cultures incubated with medium alone (figure 1). Maximal nitrite concentrations occurred at day 11 (figure 1). Of interest, nitrite accumulation was only seen in the supernatants of cell cultures from donors 3 and 4, whereas it did not occur with cells from donors 1 and 2 (figure 1). Bacterial contamination was excluded by negative culture of supernatants on blood and chocolate agar plates.

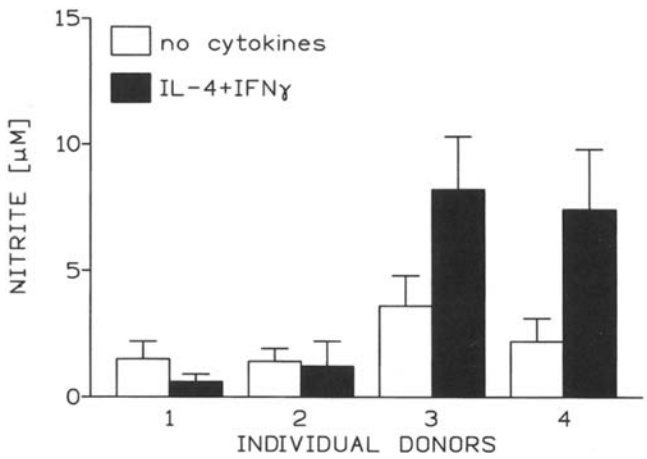

Figure 1. Nitrite generation in supernatants of 4 donors' macrophages after 11 days of culture. Human blood–derived macrophages were preincubated with interferon-γ (IFN-γ) for 48 h and then cultured in Iscove's modified Dulbecco's medium supplemented with pooled human serum and gentamicin with or without IFN-γ and recombinant human interleukin-4 (IL-4). Data are means ± SEs of 3 independent experiments.

To correlate nitrite generation with NOS activity, we investigated in parallel the metabolic parameters more specific for the NOS pathway (i.e., consumption of L-arginine with simultaneous formation of citrulline and concomitant induction of the biosynthesis of tetrahydrobiopterin, an obligate cofactor of NOS). Figure 2 shows the concentrations of 4 of 24 amino acids analyzed in macrophage culture supernatants in which >7 μM nitrite accumulated during culture with IFN-γ/IL-4 (see figure 1, donor 4). During the 10-day incubation period, there was no significant consumption of L-arginine. The concentration of L-citrulline remained below the detection limit throughout the incubation period. Ornithine concentrations were unchanged, while serine was progressively consumed during the incubation period. There was also a rapid and complete consumption of tryptophane and aspartic acid and a successive 2-fold increase of L-alanine concentrations during the 10 days (data not shown), indicating considerable metabolic activity of the human macrophages during long-term culture. There were essentially no differences in amino acid profiles in supernatants from cultures in which nitrite accumulated (donors 3 and 4) and those in which it did not (donors 1 and 2). In nitrite-

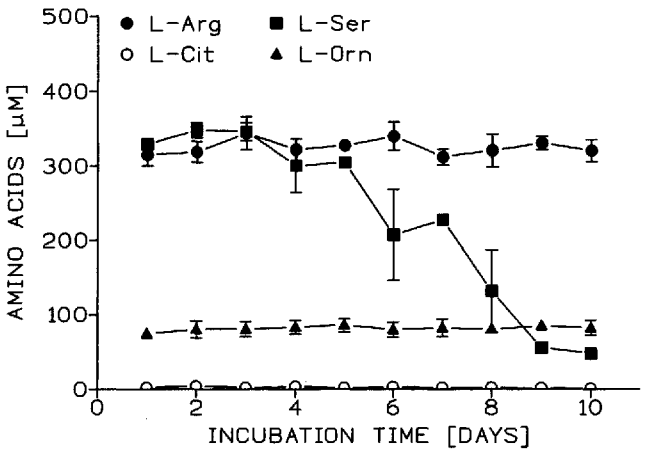

Figure 2. Changes of amino acid concentrations in human macrophage (from donor 4) supernatants. Cells were cultured in Iscove's modified Dulbecco's medium supplemented with pooled human serum, gentamicin, interferon-γ, and interleukin-4. At indicated times, aliquots of medium were removed for amino acid analysis. Of 24 simultaneously determined amino acids, 4 are shown: L-Arg = L-arginine, L-Cit = L-citrulline, L-Ser = L-serine, L-Orn = L-ornithine. Data are means ± SEs of 4 independent experiments.

Table 1. Intracellular pterins (in pmol/10^6 cells) in human macrophages after long-term exposure to recombinant human interferon-γ and interleukin-4 (IFN-γ/IL-4).

Donor, stimulus	Biopterin	Neopterin
1		
None	<0.1	1.42 ± 0.96
IFN-γ/IL-4	<0.1	7.20 ± 1.27
2		
None	<0.1	4.33 ± 0.66
IFN-γ/IL-4	<0.1	5.25 ± 0.98
3		
None	<0.1	4.04 ± 1.22
IFN-γ/IL-4	<0.1	15.80 ± 1.54
4		
None	<0.1	1.34 ± 0.49
IFN-γ/IL-4	<0.1	20.64 ± 1.45

NOTE. Human blood–derived macrophages were cultured for 11 days in Iscove's modified Dulbecco's medium supplemented with pooled human serum and gentamicin in presence or absence of IFN-γ/IL-4. Intracellular pterins were determined after acidic oxidation that converts tetrahydrobiopterin into biopterin, as described [16]. Data are means ± SEs from 3 independent experiments.

forming long-term cultures of human macrophages, neither L-arginine (5 m*M*) nor the L-arginine analogues L-NMMA (1 m*M*) and L-NNA (1 m*M*) influenced nitrite generation (data not shown), indicating that NOS activity was not involved in this process.

To test for biosynthesis of the tetrahydrobiopterin cofactor by human macrophages in long-term cultures, intracellular pterins were determined after 10 days of incubation. Neither tetrahydrobiopterin nor biopterin was detected in the cells of the macrophage culture tested (table 1). However, in the presence of IFN-γ/IL-4, there was a moderate increase of neopterin production in cells from donors 1 and 2 and an enhanced increase in cells from donors 3 and 4, neopterin being an alternate end product in the tetrahydrobiopterin synthetic pathway (table 1). Therefore, nitrite formation appeared not to be the result of tetrahydrobiopterin-dependent NOS activity.

We suspected that nitrite generation depended on the reduction of nitrate contained in the culture media; therefore, the source of nitrite generation was investigated in different tissue culture media most commonly used for the culture of peripheral blood mononuclear cells: RPMI 1640, M-199, IMDM, and MEM. These media differ considerably in their content of inorganic nitrate salts. RPMI 1640 contains 1218 μ*M* NO_3^- as $Ca(NO_3)_2$, M-199 contains 8.7 μ*M* NO_3^- as $Fe(NO_3)_3$, and IMDM contains 0.76 μ*M* NO_3^- as KNO_3; MEM does not contain nitrate salts. For the subsequent studies, media were supplemented with 5% FCS if not indicated otherwise.

Complete RPMI, the medium with the highest nitrate content, yielded 15 μ*M* nitrite after 16 days of culture (figure 3). FCS-supplemented MEM surprisingly generated nitrite as well. When MEM was supplemented with an amount of $Ca(NO_3)_2$ equal to that contained in RPMI (100 mg/L), nitrite generation increased (figure 3). When complete RPMI was supplemented with 50 mg/L ascorbic acid, the concentration contained in MEM, nitrite formation was enhanced (figure 3). Ascorbic acid as a reducing agent most probably supported nitrite generation from nitrate salts. In serum-free MEM, no nitrite accumulated

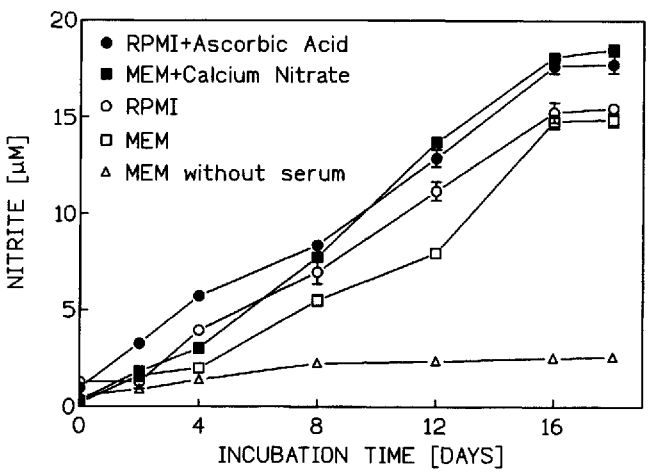

Figure 3. Kinetics of nitrite generation during long-term incubation of different cell-free culture media. RPMI 1640 (RPMI), which contains $Ca(NO_3)_2$ but no ascorbic acid, and MEM, which contains ascorbic acid but no nitrate salt, were supplemented with fetal calf serum, gentamicin, interferon-γ, and interleukin-4 to form complete media. Complete MEM was used alone and with $Ca(NO_3)_2$. Complete RPMI was used alone and with ascorbic acid. Media were incubated, and nitrite concentrations were determined at indicated times. Data are means ± SEs of 3 independent experiments.

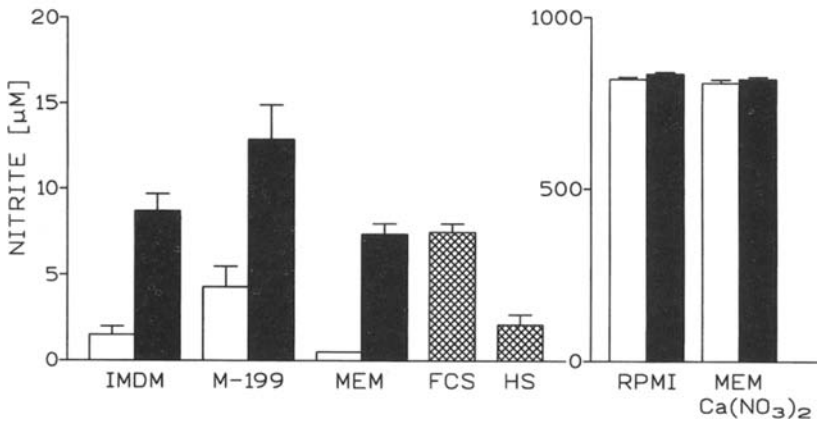

Figure 4. Nitrite generation after reduction of nitrate by nitrate reductase in different culture media and sera. RPMI 1640 (RPMI), Iscove's modified Dulbecco's medium (IMDM), Medium 199 (M-199; GIBCO, Basel, Switzerland), and MEM with and without $Ca(NO_3)_2$ were incubated with (solid bars) or without (open bars) fetal calf serum (FCS). FCS and human pooled serum (HS) were diluted in tissue culture–grade water at final concentration of 5% (hatched bars). Nitrite was measured after media and sera were incubated with nitrate reductase and NADPH. Data are means ± SEs of 4 independent experiments.

(figure 3), indicating that the source of nitrite was FCS in complete MEM.

To elucidate the chemical nature of nitrite generation in cell-free culture media, we further related nitrite formation to reduction of nitrate. As shown in figure 4, serum-free culture media yielded micromolar amounts of nitrite upon enzymatic nitrate reduction by an exogenous nitrate reductase according to their formulated nitrate content. Of note, MEM, the medium with no nitrate salt, did not yield nitrite after incubation with nitrate reductase. When supplemented with $Ca(NO_3)_2$ at the concentration formulated in RPMI, nitrite was found as expected. Media supplemented with 5% FCS generated more nitrite by nitrate reductase; the amounts corresponded to the nitrite formed upon reduction of 5% FCS in nitrate-free water. Considerably less nitrite was formed from 5% pooled human serum.

In summary, enzymatic reduction of culture media with or without FCS yielded nitrite in concentrations corresponding to nitrate concentrations in the different media as well as in sera (figure 4). This indicated that FCS could be the source of nitrite in MEM, which by itself does not contain nitrate (figures 3, 4). Without enzymatic reduction, both fresh media and sera contained <1 μM nitrite (not shown).

In a next set of experiments, further confirmation was sought that nitrite accumulates by reduction of nitrate to nitrite and not by the L-arginine–NOS pathway in cell-free culture media. After incubation of medium, when nitrite had accumulated and nitrate already present in the medium was reduced to nitrite, the amount of nitrite obtained was the same as that obtained without incubation for nitrite accumulation (figure 5). In contrast, when nitrite formation depended on NOS (as is the case in aerobic cultures of SMCs stimulated with IFN-γ/LPS), nitrite formed through the L-arginine–NOS pathway contributed to the total amount of nitrite measured after treatment of supernatants by nitrate reductase. As further support of our hypothesis, we found that in the absence of O_2, NOS activity did not contribute to nitrite formation in SMCs (figure 5).

In addition, in the absence of O_2, no citrulline was produced and no L-arginine was consumed in activated SMCs. In human monocytes stimulated with IFN-γ/IL-4, neither aerobic nor anaerobic cultures supported the accumulation of nitrite, L-arginine consumption, or citrulline production (table 2). The long-term incubation of cell-free medium resulted in an accumulation of nitrite under both anaerobic and aerobic conditions without changes in L-arginine and citrulline concentrations.

Since we and others had observed that nitrite accumulates preferentially in the presence of IFN-γ/IL-4, we studied whether these cytokines themselves had nitrate reductase activity. Indeed, when media differing in their nitrate formulation

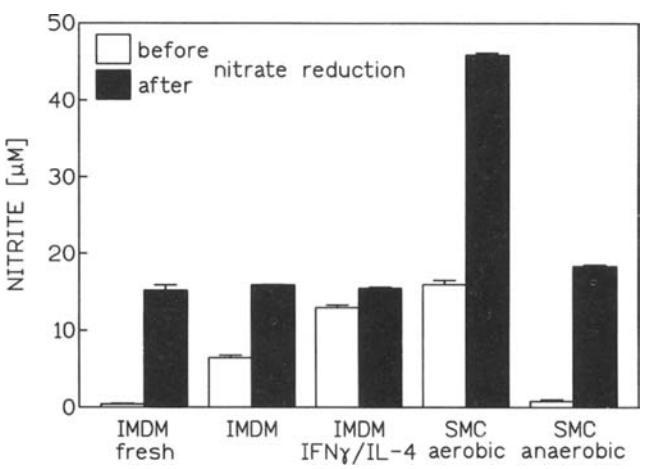

Figure 5. Nitrite determination in cell-free media and smooth muscle cell (SMC) culture supernatants before and after total nitrite and nitrate measurements. Nitrite-free Iscove's modified Dulbecco's medium (IMDM) was supplemented with fetal calf serum and gentamicin. There was significant nitrite accumulation after incubating IMDM for 12 days ($P < .001$). Supplementation of medium with interferon-γ and interleukin-4 (IFN-γ/IL-4) augmented nitrite accumulation ($P < .001$). Amount of nitrite in medium was same after nitrate reduction whether nitrite had or had not accumulated prior to nitrate reduction. During 48-h incubation, no spontaneous nitrite accumulation occurred (not shown). Within 48 h, rat SMC activated with lipopolysaccharide and rat recombinant IFN-γ for induction of inducible NOS produced nitrite that was additive to total concentration measured after reduction of nitrate. Data are means ± SEs of 3 independent experiments.

Table 2. Nitrite generation, arginine turnover, and citrulline produced in human macrophages, rat aortic smooth muscle cells (SMCs), and cell-free medium incubated under aerobic and anaerobic conditions.

Sample, condition	Nitrite generated (μM)	Arginine consumed (μM)	Citrulline produced (μM)
Macrophage			
Aerobic	<0.5	<2	<2
Anaerobic	<0.5	<2	<2
SMCs			
Aerobic	14.20 ± 0.92	30.1 ± 4.0	22.5 ± 2.5
Anaerobic	<0.5	<2	<2
Medium			
Aerobic	5.08 ± 0.45	<2	<2
Anaerobic	4.12 ± 0.43	<2	<2

NOTE. Human blood–derived macrophages were cultured for 12 days in Iscove's modified Dulbecco's medium (IMDM) supplemented with 25% pooled human serum, gentamicin, recombinant human interferon-γ (IFN-γ), and recombinant human interleukin-4. Identically supplemented cell-free IMDM was also incubated for 12 days. SMCs were cultured in IMDM with fetal calf serum, gentamicin, lipopolysacchride, and rat recombinant IFN-γ for 48 h. Data are means ± SEs from 3 independent experiments.

were incubated with IL-4/IFN-γ, a heat-labile activity-reducing nitrate was observed (figure 6).

Discussion

During the last decade, a high-output NOS system has been characterized in murine macrophages [1, 20, 21]. The existence of a parallel enzyme system in human phagocytes could, however, not be established despite considerable efforts [reviewed in 22]. In the present study, we reproduced previous observations on nitrite generation in long-term cultures of IFN-γ/IL-4–treated macrophages from some subjects [9–15]; however, we found that nitrite generation in culture supernatants of human macrophages is not a result of NOS activity. This is because these cells do not consume L-arginine, the substrate of NOS, or produce L-citrulline, the coproduct (along with nitric oxide) of NOS (figure 2). Moreover, biosynthesis of tetrahydrobiopterin, an obligate cofactor of NOS, was not induced during nitrite generation (table 1). In further contrast to the rodent macrophage system [23], human macrophages do not produce L-ornithine or regenerate L-arginine from L-citrulline as an endogenous supply of substrate for the high-output NOS [16]. Because of a lack of urea cycle enzymes, human macrophages did not generate ornithine and urea from L-arginine during long-term culture (figure 2) [16].

Since nitrite accumulation in human monocyte cultures stimulated with IFN-γ/IL-4 appeared not to depend on the L-arginine–NOS pathway, we wondered about the source of nitrite under such study conditions. We first found that nitrite also accumulated in cell-free medium and that the amount of its formation depended on the formula of the culture medium, particularly of its nitrate content (figure 3). In addition, sera were a considerable source of nitrate (figure 4). Of note, previous studies reporting nitrite after treatment of culture supernatants with exogenous nitrate reductase (the so-called total nitrite/nitrate measurement) massively underestimated nitrite/nitrate concentrations [18], presumably because of limiting NADPH cofactor concentrations in the assay system (figure 4); indeed, their concentrations did not even represent the nitrate formulated in their medium. By using the total nitrite/nitrate measurement and comparing it with the nitrite measurement without nitrate reduction, we demonstrated that nitrite accumulation in cell-free medium (in contrast to NOS-expressing cell cultures) depended on the reduction of nitrate present in the medium (figure 5). Accordingly, by studying L-citrulline production and L-arginine consumption as more specific parameters for NOS activity, we found corresponding changes in amino acid concentrations (table 2). Furthermore, NOS activity in NOS-expressing SMCs, but not nitrate reduction, was abolished under anaerobic conditions.

Because IFN-γ/IL-4 treatment was associated in previous studies [9–15] and the present study with nitrite formation, we wondered whether these cytokines display nitrate reductase activity. We found that the IFN-γ/IL-4 combination does indeed have heat-labile nitrate reductase activity, explaining the increased nitrite accumulation when these cytokines are added to nitrate-containing media (figure 6).

In addition to these findings, the present study elaborates tools to investigate nitrite accumulation in cell cultures and its relation to the L-arginine–NOS pathway, including important control experiments that could prevent future pitfalls.

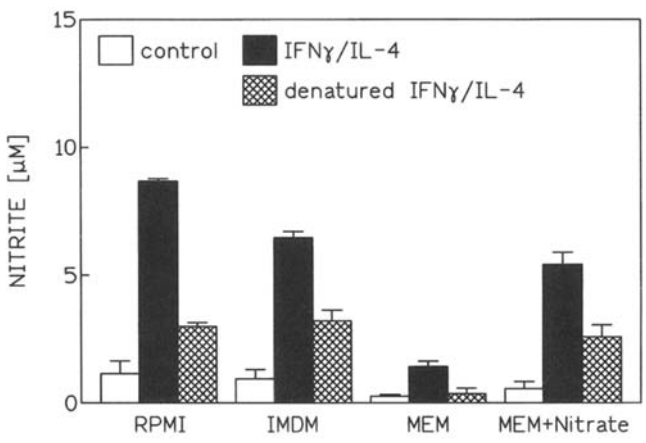

Figure 6. Promotion by human recombinant interferon-γ and interleukin-4 (IFN-γ/IL-4) of spontaneous nitrite accumulation in media. Cell-free media (RPMI 1640 [RPMI], Iscove's modified Dulbecco's medium [IMDM], MEM, and MEM with nitrate) supplemented with fetal calf serum and gentamicin were incubated with or without IFN-γ/IL-4 before assessment of nitrite concentrations. IFN-γ/IL-4 enhanced nitrite accumulation ($P < .01$). Heightened nitrite accumulation was blunted by heat-inactivation of cytokines for 5 min at 95°C ($P < .01$). Data are means ± SEs of 3 independent experiments.

In conclusion, our extensive studies confirm that in human monocyte cultures, nitrite can accumulate if the cells are incubated with IFN-γ/IL-4 for several days. By using more in-depth studies including parameters specific for the NOS pathway, anaerobic control cultures, tetrahydrobiopterin determinations, and a comparison of total nitrite/nitrate measurement with nitrite accumulation, we can show that the NOS pathway is not involved in this accumulation. Nitrite is formed by reduction of nitrate formulated in media and naturally present in sera. IFN-γ/IL-4 appear to promote nitrite formation in media due to nitrate-reducing activity contained in these cytokines.

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