Interferon-γ and Tumor Necrosis Factor Synergize to Induce Nitric Oxide Production and Inhibit Mitochondrial Respiration in Vascular Smooth Muscle Cells

Yong-jian Geng, Göran K. Hansson, and Elisabeth Holme

Nitric oxide (NO) is an important signal substance in cell–cell communication and can induce relaxation of blood vessels by activating guanylate cyclase in smooth muscle cells (SMCs). NO is synthesized from L-arginine by the enzyme NO synthase, which is present in endothelial cells. It was recently shown that SMCs may themselves produce NO or an NO-related compound. We have studied NO production and its effects on energy metabolism in cultured rat aortic smooth muscle cells. It was observed that the cytokines, interferon-γ and tumor necrosis factor-α, synergistically induced an arginine-dependent production of NO in these cells. This was associated with an inhibition of complex I (NADH:ubiquinone oxidoreductase) and complex II (succinate:ubiquinone oxidoreductase) activities of the mitochondrial respiratory chain, suggesting that NO blocks mitochondrial respiration in these cells. Lactate accumulated in the media of the cells, implying an increased anaerobic glycolysis, but there was no reduction of viability. An NO-dependent inhibition of mitochondrial respiration and a switch to anaerobic glycolysis would reduce the energy production of the SMCs. This would in turn reduce the contractile capacity of the cell and might represent another NO-dependent vasodilatory mechanism. It could be of particular importance in inflammation, since cytokines released by inflammatory cells may induce autocrine NO production in SMCs. (*Circulation Research* 1992;71:1268–1276)

KEY WORDS • **interferon gamma** • **mitochondria** • **nitric oxide** • **vascular smooth muscle** • **tumor necrosis factor**

Nitric oxide (NO) accounts for the activity of the endothelium-derived relaxing factor, which causes smooth muscle cell (SMC) relaxation through a cGMP-dependent mechanism.[1–4] NO is also produced in the brain, where it is thought to mediate the action of the excitatory transmitter glutamate,[2,5–7] and in activated macrophages, which produce NO that inhibits the mitochondrial respiration of target cells, leading to cytotoxicity.[8–12]

NO is formed from L-arginine in an O_2-dependent reaction.[2] Several different NO-synthesizing enzymes have recently been identified.[7,8,13] One group of enzymes is constitutively expressed in endothelial cells and neurons, whereas another enzyme is inducible in macrophages. All enzymes catalyze the conversion of L-arginine to NO and citrulline with NADPH as a cofactor. The soluble brain/endothelial enzyme, type I NO synthase, was recently cloned and sequenced. It is Ca^{2+}/calmodulin–dependent and inhibited by the arginine analogues N^G-monomethyl-L-arginine (L-NMMA) and N^G-nitro-L-arginine-methyl ester (L-NAME).[2,12] The macrophage enzyme, type II NO synthase, is Ca^{2+} independent and is inhibited more strongly by L-NMMA than by L-NAME.[8,12] Expression of NO synthase type II is not constitutive but inducible by cytokine stimulation.[2,8] Cloning of an inducible (type II) NO synthase from murine macrophages[14,15] has revealed substantial homology to the constitutive enzyme and also shown that interferon-γ (IFN-γ) and lipopolysaccharide induce its expression on the transcriptional level.[14]

It was recently shown that SMCs not only respond to endothelium-derived NO but are able to produce their own NO. Stimulation with the cytokines, IFN-γ and tumor necrosis factor-α (TNF-α), induce NO and cGMP production in these cells.[16] The pattern of induction and inhibition of this activity suggests that SMCs can express NO synthase type II.[16–18] When produced by NO synthase type II–expressing macrophages, NO binds to nonheme iron–containing enzyme clusters of the respiratory chain in target cells and block their aerobic energy metabolism.[8–10,19] A similar effect in SMCs might add to the vasodilating effect by reducing the production of energy-rich nucleotides that are necessary for muscle contraction.

Figure 1 contains a schematic representation of the metabolism of glucose, which is degraded to pyruvate that may be used as a substrate in the citric acid cycle, with subsequent oxidation in the mitochondrial respira-

From the Department of Clinical Chemistry, Gothenburg University, Sahlgren's Hospital, Gothenburg, Sweden.

Supported by the Swedish Medical Research Council (projects 6816 and 0585), the Swedish Heart-Lung Foundation, the Swedish National Association Against Rheumatism, and the funds of Gothenburg University.

Address for correspondence: Dr. Göran K. Hansson, Gothenburg University, Department of Clinical Chemistry, Sahlgren's Hospital, S-413 45 Gothenburg, Sweden.

Received March 25, 1992; accepted July 17, 1992.

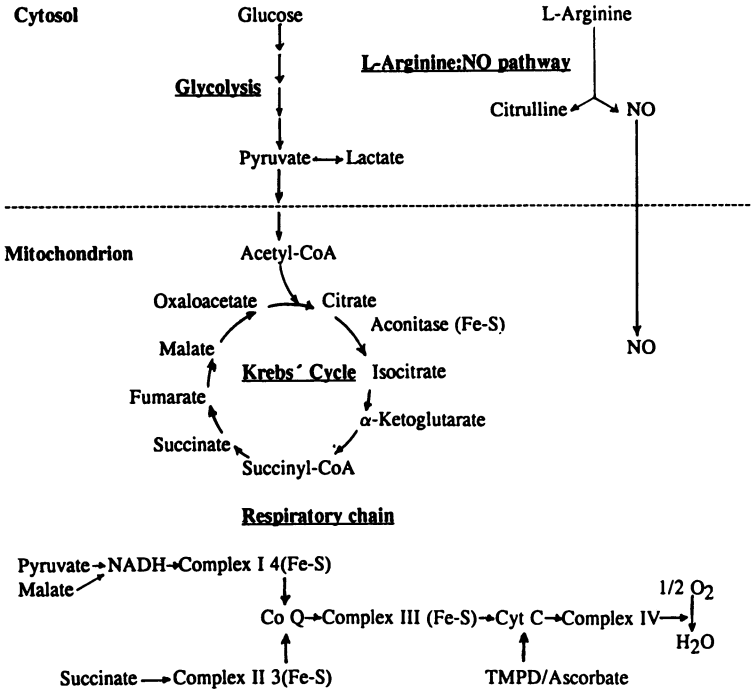

FIGURE 1. *Schematic representation of the glucose metabolism, respiratory chain, and L-arginine:nitric oxide (NO) pathway. CoA, coenzyme A; TMPD, tetramethylphenylenediamine.*

tory chain. This process is dependent on oxygen and will generate 36 ATP molecules per glucose molecule that enters the pathway. Alternatively, pyruvate may be reduced to lactate, producing a total of 2 ATP molecules per glucose molecule. Several of the enzymes in the aerobic pathway contain nonheme iron-sulfur clusters to which NO could bind; this would result in an inhibition of aerobic glucose metabolism and a reduced production of ATP.[9]

We have studied the effects of cytokines on autocrine NO production and its effects on glycolysis and mitochondrial respiration in cultured rat aortic SMCs. Our data show that IFN-γ and TNF synergistically induce NO production from L-arginine and that this is accompanied by an inhibition of mitochondrial respiration and an increased lactate production. These findings suggest that the L-arginine–NO pathway is involved in the regulation of energy metabolism in SMCs. This may represent an additional NO-mediated mechanism for the modulation of vascular contractility.

Materials and Methods

Reagents

Recombinant rat IFN-γ was purchased from Holland Biotechnology, Leiden, The Netherlands. Its specific activity was 4×10^6 units/mg protein. Recombinant human IFN-γ and human and murine TNF-α (which both react with the rat TNF receptor) were gifts from Genentech, Inc., South San Francisco, Calif. Sulfanilamide and *N*-(1-naphthyl)ethylenediamine hydrochloride (N-1-NED) were obtained from Merck, Darmstadt, FRG. L-NMMA and L-NAME were from Calbiochem Corp., La Jolla, Calif., and Serva, Heidelberg, FRG, respectively. Digitonin, pyruvate, malate, succinate, rotenone, tetramethylphenylenediamine (TMPD), antimycin A, and ascorbate were purchased from Sigma Chemical Co., St. Louis, Mo. Nitric oxide gas (500 ppm in nitrogen) was obtained from AGA, Gothenburg, Sweden.

Smooth Muscle Cell Culture

Vascular SMCs were isolated from the aorta of 6-week-old Sprague-Dawley rats by collagenase digestion and cultured in RPMI-1640 medium (GIBCO, Paisley, Scotland) supplemented with 10% fetal calf serum (FCS) and antibiotics as previously described.[20] Cultures at passages 4–10 were used for the experiments. They were identified as SMCs by their typical growth pattern of "hills and valleys" and by immunofluorescence using a monoclonal antibody to α-smooth muscle actin[21] (a gift from Dr. G. Gabbiani, University of Geneva, Switzerland). Confluent cultures were trypsinized, and the cells were plated at a density of 2×10^4/ml for experiments. Cell viability was determined using trypan blue exclusion.[22]

DNA Synthesis

SMCs were grown in 24-well plates (Nunc, Roskilde, Denmark) and synchronized in the quiescent G_0 phase of the cell cycle by 48 hours of serum starvation in 0.5% FCS. They were then stimulated to enter the cell cycle by exposure to regular culture medium containing 10% FCS[20]. Cytokines were added as described in figure legends. [^{3}H]Thymidine (Amersham, UK) was added after 32 hours, and the cells were trypsinized and harvested on glass filters 48 hours after addition of RPMI/10% FCS. Radioactivity was determined by liquid scintillation counting.

Cell Protein

Cells in 96-well microtiter plates were fixed in 4% formaldehyde, stained for protein with Amido Black B, and rinsed with water. The protein–dye complex was dissolved in 0.2 M NaOH and read at 620 nm in an SLT EIA microtiter spectrophotometer.[20]

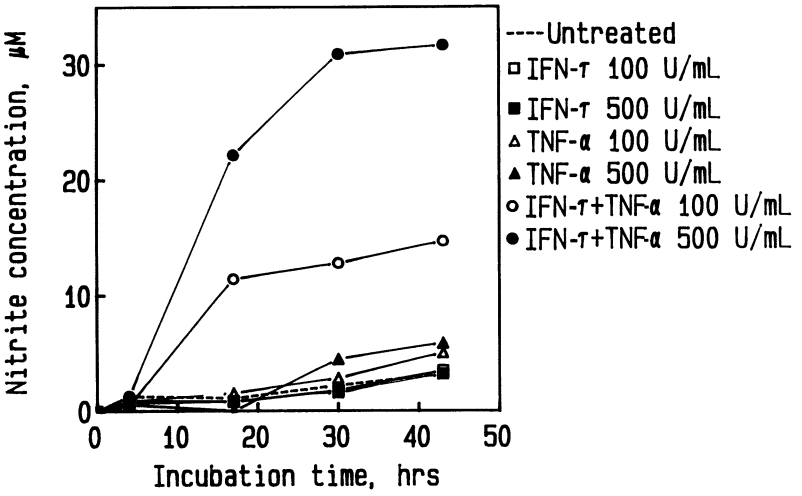

FIGURE 2. *Time course of nitrite concentration. Interferon-γ (IFN-γ) and tumor necrosis factor-α (TNF-α) synergistically induce nitrite accumulation in smooth muscle cell cultures. Smooth muscle cells were grown in microtiter plates and treated at subconfluent density with recombinant cytokines at the indicated doses. Media were assayed for nitrite after different time intervals. Symbols indicate means of six experiments, with coefficients of variation <8%.*

Nitrite Assay

NO production was assessed by measuring its stable end product, nitrite, in the culture media using a modification of Bell's method, which relies on the diazotization of sulfanilic acid by nitrite at acidic pH and its coupling to N-1-NED.[4] Spent media from SMC cultures were incubated with 2.9 mM sulfanilic acid, 2 M HCl, and 0.2 M N-1-NED at room temperature for 30 minutes, and the colored product was quantitated at 538 nm. Nitrite release from SMCs was determined by subtracting nitrite levels in cell-free medium from those in medium with cells.

Lactate and Glucose Analysis

Lactate and glucose were assayed in aliquots of SMC culture media. Perchloric acid (3%) was added to precipitate proteins, and supernatants were collected after centrifugation and used for enzymatic assays as previously described.[23,24] Lactate production and glucose consumption by the cells over a specified time was assessed from the changes in concentration as follows:

lactate production (millimolar)=lactate concentration after incubation−concentration before incubation;

glucose consumption (millimolar)=glucose concentration before incubation−concentration after incubation.

Analysis of Mitochondrial Respiration

SMCs were collected by trypsinization and washed in ice-cold phosphate-buffered saline. They were permeabilized with digitonin essentially as described.[25] In brief, 2×10^7 cells were washed in respiratory medium containing (mM) sucrose 250, HEPES 20, pH 7.2, $MgCl_2$ 10, K_2HPO_4 2, and EGTA 1 and suspended in 10 ml ice-cold respiratory medium with 0.007% digitonin. The efficiency of permeabilization was tested by trypan blue exclusion, which showed permeability >97% in each cell preparation. Permeabilized cells were centrifuged at 400*g* for 5 minutes to remove the cytosol. The pellet was suspended in 1 ml respiratory medium with 1 mM ADP and 0.5% bovine serum albumin on ice. Cell suspensions (100 μl) were used for polarographic measurements of oxygen consumption at 25°C with a Clark electrode in a 600-μl magnetic stirred cell (Diamond Electro-Tech, Ann Arbor, Mich.). Substrates and inhibitors were added consecutively to measure different parts of the respiratory chain (Figure 1): 5 mM pyruvate and 2.5 mM malate for NADH:ubiquinone oxidoreductase (complex I)–dependent oxidation, 3 μM rotenone followed by 5 mM succinate for succinate:ubiquinone oxidoreductase (complex II)–dependent oxidation, and 1 μM antimycin A followed by 0.5 mM TMPD and 5 mM ascorbate for cytochrome *c* oxidase (complex IV) activity. In some experiments, the cGMP analogue, 8-bromo-cGMP (Sigma), was added to the culture medium at 1 mM and incubated with cells for 24 hours before permeabilization and assay of mitochondrial respiration.

Exposure to Exogenous NO

SMCs were trypsinized and incubated in respiratory medium at 10^7 cells/ml in 12-ml plastic test tubes. NO gas (40 ppm in nitrogen with 21% air) was bubbled through the cell suspension for 10 minutes. Cells were kept on ice throughout the exposure to NO, and viability was >85% after NO exposure. The cells were then permeabilized with digitonin, and mitochondrial respiration was assessed by oximetry as described above.

Statistics

Linear regression was evaluated using the least-squares method. Student's *t* test was used to analyze differences between means.

Results

IFN-γ+TNF-α Induces Nitrite Production in SMCs

NO production in SMCs was evaluated by analysis of its stable metabolite, nitrite, in culture media. Nitrite is a major oxidation product of NO and therefore a good indicator of the amount of NO formed in a biological system.[26] As shown in Figure 2, rat aortic SMCs in RPMI-1640 medium supplemented with 10% FCS (which contains approximately 2 mM L-arginine) produced little nitrite. Neither IFN-γ nor TNF-α alone induced nitrite accumulation in SMC culture media (Figure 2). However, when the cells were incubated with a combination of IFN-γ and TNF-α, they released significant amounts of nitrite into the medium (Figure 2). Cytokine-induced nitrite production was dose and time dependent, and treatment of SMCs with IFN-γ+TNF-α at 100 units/ml each for 18 hours increased

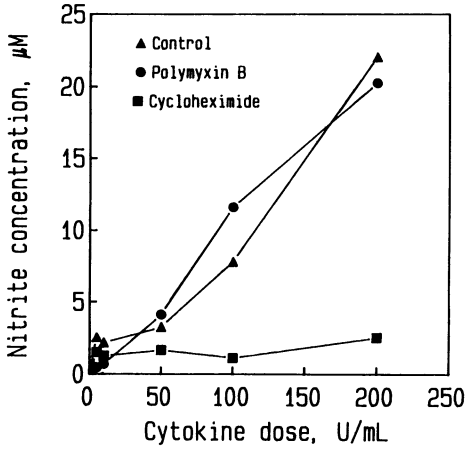

FIGURE 3. *Graph showing that cytokine-induced nitrite accumulation in smooth muscle cells is dependent on protein synthesis and is not abolished by an endotoxin inhibitor. Smooth muscle cells were treated for 12 hours with interferon-γ plus tumor necrosis factor-α at the indicated doses, and nitrite concentrations were determined in the culture medium. Cycloheximide (10 μg/ml) and polymyxin B (50 μg/ml) were added together with cytokines. Symbols indicate means of six experiments. All coefficients of variation were <8%.*

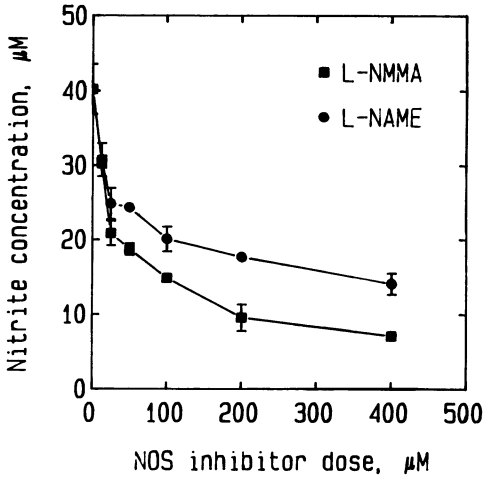

FIGURE 4. *Graph showing that cytokine-induced nitrite accumulation is dependent on L-arginine. L-NMMA,* N^G*-monomethyl L-arginine; L-NAME,* N^G*-nitro-L-arginine-methyl ester; NOS, nitric oxide synthase. Smooth muscle cells were treated for 12 hours with interferon gamma plus tumor necrosis factor-α (200 units/ml each) in the presence or absence of the arginine analogues L-NMMA and L-NAME. Symbols indicate means of six experiments; all coefficients of variation were <8%.*

nitrite concentration in the medium to approximately 10 times that in control medium (Figure 2).

Nitrite production in IFN-γ+TNF-α–treated SMCs was completely inhibited by adding the protein synthesis inhibitor cycloheximide (10 μg/ml), indicating that nitrite production was dependent on cytokine-induced protein synthesis (Figure 3). Nitrite accumulation was accompanied by expression of the IFN-γ–inducible gene, RT1D (rat class II major histocompatibility protein; data not shown). Nitrite production was unaffected by the endotoxin inhibitor polymyxin B (Figure 3). Furthermore, nitrite production required the simultaneous addition of IFN-γ and TNF-α, which also argues against the possibility that an endotoxin contamination could account for the observed effects.

To determine whether nitrite was derived from the L-arginine–NO pathway, the L-arginine analogues L-NMMA and L-NAME were added to the cultures together with the cytokines. Figure 4 shows that either analogue diminished nitrite production in cytokine-treated SMCs, indicating that the nitrite was derived from L-arginine. At equivalent doses, L-NMMA was, however, a more effective inhibitor than L-NAME (Figure 4). The 50% inhibitory dose was 39 μM for the former compared with 92 μM for the latter. This pattern is similar to that observed for the macrophage-type cytokine-inducible NO synthase[8] but contrasts with the constitutively expressed brain/endothelial NO synthase,[5,8] suggesting that the cytokine-induced NO synthase of SMCs is similar to that of macrophages.

IFN-γ+TNF-α Inhibits Mitochondrial Respiration in SMCs

We hypothesized that autocrine NO production might affect the intermediary metabolism of SMCs and therefore evaluated glucose consumption, mitochondrial respiration, and lactate production in these cells. Figure 1 outlines the metabolism of glucose, which is degraded to pyruvate that may be used as a substrate in the citric acid cycle with subsequent oxidation in the mitochondrial respiratory chain. Enzyme complex I, complex II, and ubiquinol:cytochrome *c* reductase (complex III) of the mitochondrial respiratory chain contain nonheme iron sulfur clusters, which form nitrosyl complexes with NO. This is known to result in an inhibition of respiration in NO-producing macrophages and their target cells.[9,10] Nitrite accumulation was of the same magnitude in cytokine-treated SMCs as in macrophages, and it was therefore reasonable to hypothesize that NO production might inhibit mitochondrial respiration also in SMCs. We tested this by treating SMCs with IFN-γ+TNF-α for 24 hours and then assessing mitochondrial respiration after digitonin permeabilization of the cells.

Figure 5 shows representative oxygen tracings of cytokine-treated and control SMCs. After removal of endogenous substrates by washing, permeabilized SMCs consumed little oxygen without addition of the substrates for mitochondrial respiration. Addition of pyruvate, malate, ADP, and inorganic phosphate as substrates for enzyme complex I resulted in a significant linear oxygen consumption in control SMCs (Figure 5, tracing B). The respiratory rate was reduced by approximately 50% in SMCs treated with IFN-γ+TNF-α (200 units/ml each), suggesting a block in complex I respiration (Figure 5, tracing A; Table 1). When complex I was inhibited by rotenone, the addition of succinate initiated complex II respiration (Figure 5; for overview, see Figure 1). This response was also significantly reduced in cells treated with IFN-γ+TNF-α, implying complex II as a second blocking site (Figure 5). With inhibition of succinate respiration by antimycin A, oxygen consumption was resumed by adding TMPD and ascorbate, reflecting complex IV activity (Figure 5). The respira-

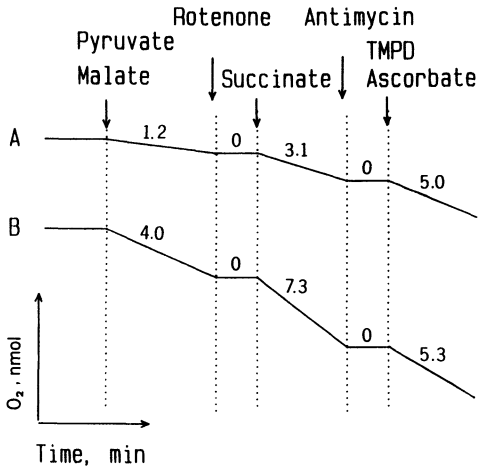

FIGURE 5. *Time course showing that cytokine treatment blocks the respiratory chain in smooth muscle cells. TMPD, tetramethylphenylenediamine. Cells were treated with interferon-γ plus tumor necrosis factor-α (200 units/ml each) for 24 hours, permeabilized, and assayed for mitochondrial respiration using an oxygen electrode as described. Representative oxygen tracings are shown of cytokine-treated (tracing A) and untreated (tracing B) smooth muscle cells. Oxygen concentration is plotted vs. time of the experiment. Substrates and inhibitors of the respiratory chain enzyme complexes are indicated, and oxygen consumption (in nanomoles of oxygen per minute per 10^6 cells) is shown for each complex.*

tory rate of complex IV was only slightly reduced in cytokine-treated SMCs compared with control cells (Figure 5).

Table 1 shows oximetric data of cytokine effects on mitochondrial respiration in a series of six experiments. A significant inhibition of complexes I and II of the respiratory chain was found only in IFN-γ+TNF-α cotreated SMCs, which had been shown to produce large amounts of nitrite from L-arginine (see above). Treatment with a combination of IFN-γ and TNF-α at 200 units/ml led to a 50% reduction in complex I respiration with pyruvate/malate as substrates and to a similar reduction of complex II respiration with succinate as a substrate. At the same dose, TNF-α alone did not significantly alter the respiratory rates, and IFN-γ induced a moderate reduction of complex II but not complex I respiration (Table 1).

TABLE 1. Inhibition of Mitochondrial Respiration in Rat Vascular Smooth Muscle Cells Treated With Cytokines

Treatments	Oxygen consumption (nmol O_2/min per 10^6 cells)		
	Pyruvate/malate	Succinate	TMPD/ ascorbate
Untreated	3.19±0.39	5.85±0.55	5.77±0.48
IFN-γ	3.10±0.42	4.23±0.54	4.48±0.77
TNF-α	3.58±1.37	5.20±0.19	5.82±0.53
IFN-γ+TNF-α	1.68±0.16*	3.09±0.36*	4.22±0.33*

TMPD, tetramethylphenylenediamine; IFN-γ, interferon-γ; TNF-α, tumor necrosis factor-α. Values are mean±SEM.

Smooth muscle cells (n=6) were incubated with cytokines (200 units/ml each) for 24 hours, permeabilized, and assayed for mitochondrial respiration with an oxygen electrode. Pyruvate/malate represents complex I respiration, succinate is complex II, and TMPD/ascorbate indicates complex IV activity.

*Respiration significantly reduced from control (untreated) value (p<0.05).

TABLE 2. Effects of Cytokines, 8-Bromo-cGMP and Nitric Oxide Gas on Mitochondrial Respiration in Rat Vascular Smooth Muscle Cells

Treatment	Complex I/complex IV (% of control)
Untreated	100
IFN-γ	106
TNF-α	95
IFN-γ+TNF-α	61
NO gas	41
8-Bromo-cGMP	97

IFN-γ, interferon-γ; TNF-α, tumor necrosis factor-α; NO, nitric oxide.

Experiments were performed as described in Figure 5, Table 1, and "Materials and Methods."

That NO selectively blocked the earlier complexes in the mitochondrial respiratory chain was illustrated by calculating the ratio of complex I/complex IV respiration. It was 0.65 in untreated control SMCs, 0.69 in cells treated with IFN-γ alone, and 0.62 in TNF-α–treated cells. In contrast, the complex I/complex IV ratio in cells treated with the combination of IFN-γ+TNF-α was reduced to 0.39, i.e., 61% of the control level (Table 2).

To confirm that the cytokine-inducible effect on mitochondrial respiration can be explained by NO production, we analyzed mitochondrial respiration after exposure of SMCs to authentic NO gas. This resulted in a 50% reduction of complex I respiration and a 90% reduction of complex II respiration, whereas complex IV respiration was only reduced by 6.0%. The complex I/complex IV ratio was reduced to 41% of the level in untreated control SMCs (Table 2). In contrast, 8-bromo-cGMP did not reduce mitochondrial respiration to any significant extent (Table 2). This supports the conclusion that IFN-γ+TNF-α inhibits mitochondrial respiration by inducing NO production and that this effect is not mediated via guanylate cyclase.

IFN-γ+TNF-α Induce NO-Dependent Lactate Accumulation in SMC Cultures

Dysfunction of the respiratory chain prevents complete combustion of glucose. Its metabolism is therefore arrested after anaerobic glycolysis, the end-product of which is lactate (Figure 1). Therefore, we evaluated the metabolic significance of the reduced mitochondrial respiration in cytokine-treated cells by analyzing lactate in culture media. There was a dose- and time-dependent accumulation of lactate in IFN-γ+TNF-α–treated SMCs (Figure 6). In contrast, treatment with either cytokine alone did not induce lactate accumulation even at doses up to 3,000 units/ml (data not shown).

The kinetics of lactate production in cytokine-treated SMCs (data not shown) was similar to that observed for nitrite production as shown above. A linear regression analysis showed that the lactate concentration in culture media of IFN-γ+TNF-α–treated cells paralleled its nitrite concentration (Figure 7), which implies a link between the arginine–NO pathway and anaerobic gly-

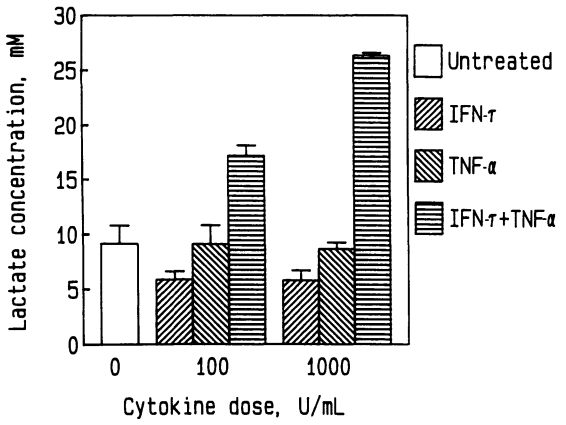

FIGURE 6. *Bar graph showing that cytokine treatment induces lactate accumulation in smooth muscle cells. IFNγ, interferon-γ; TNF-α, tumor necrosis factor-α. Cells were treated with IFN-γ or TNF-α at the indicated doses for 24 hours, and lactate concentrations were assayed in culture media. Bars indicate mean±SEM* (n=6). *Cultures treated with IFN-γ+TNF-α at either dose showed significantly higher lactate concentrations than untreated cultures* ($p<0.01$).

colysis. This was further addressed by examining the effect of the NO synthase inhibitor, L-NMMA, on lactate production. Table 3 shows that L-NMMA treatment significantly inhibits lactate production in IFN-γ+TNF-α–treated SMCs.

Anaerobic glycolysis is a less efficient mechanism for production of energy-rich nucleotides than mitochondrial oxidation. This might lead to compensatory increases in glucose uptake; therefore, we evaluated glucose consumption in cytokine-treated SMCs. Glucose concentration was determined in culture media before and after 48 hours of stimulation of the cells with IFN-γ and TNF-α. Ninety to 95% of the glucose available in the culture medium (11 mmol/l) was consumed during a 48-hour incubation. Only modest effects on glucose consumption were observed in cytokine-treated cells (Table 3). TNF-α increased glucose consumption, IFN-γ reduced it, and a combination of the two resulted in glucose levels that were similar to those obtained in cultures treated with TNF-α alone. Therefore, the cells did not compensate for the block in mitochondrial respiration by an increased glucose consumption. Instead, our data suggest that almost all glucose was degraded to lactate in cells treated with IFN-γ+TNF-α. The ratio of lactate production to glucose consumption was approximately 1 in control cells and virtually unchanged in cells treated with either IFN-γ or TNF-α (Table 3). However, treatment with IFN-γ+TNF-α increased the ratio to approximately 2 (Table 3). Since anaerobic glycolysis produces two lactate molecules for each glucose molecule, our data suggest that most of the internalized glucose was converted into lactate in NO-producing cells. This increase in anaerobic glycolysis was dependent on the L-arginine–NO pathway, since addition of L-NMMA could reverse the effect of IFN-γ+TNF-α (Table 3).

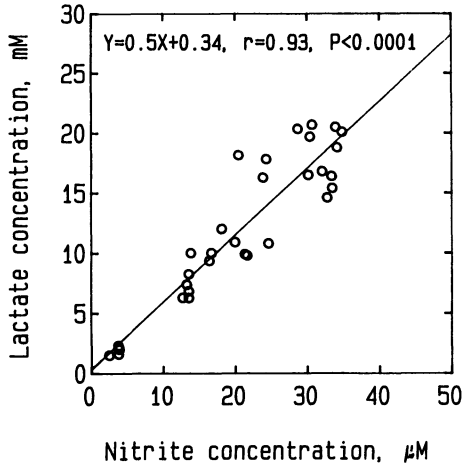

FIGURE 7. *Plot showing that lactate concentration correlates with nitrite concentration in the culture media of smooth muscle cells. The linear regression analysis was based on analyses of nitrite and lactate in aliquots of culture media from cytokine-treated and control smooth muscle cells as described in Figures 2 and 6. The equation for the regression line and the correlation coefficient (r) are indicated in the figure. Y is millimolar lactate concentration, and X is micromolar nitrite concentration.*

NO Production Does Not Affect Cellular Viability

The metabolic changes in cytokine-treated cells could reflect specific effects of NO on enzyme complexes involved in mitochondrial respiration, but they might alternatively represent the loss of enzymatic activities in a dying cell. Therefore, we evaluated the viability of cytokine-treated SMCs, both by dye exclusion and proliferative capacity. Table 4 shows that treatment with IFN-γ+TNF-α at 100 units/ml for 72 hours does not affect trypan blue exclusion. At doses of 1,000 units/ml, there was a moderate reduction in viability after 72 hours of treatment (Table 4). This indicates that at levels that block mitochondrial respiration, IFN-γ+TNF-α does not induce cell death.

SMC replication is directly regulated by IFN-γ[18] but is also sensitive to cytotoxic effects. IFN-γ significantly inhibits [^{3}H]thymidine incorporation when quiescent SMCs are stimulated to enter the cell cycle by growth factor–containing serum (Figure 8). TNF-α, on the other hand, induces a moderate increase in [^{3}H]thymidine incorporation. Cultures treated with both IFN-γ and TNF-α exhibit a reduced [^{3}H]thymidine incorporation, which is identical in size to the one induced by IFN-γ alone. The effects of NO on cell replication were evaluated by determining [^{3}H]thymidine incorporation in the presence of the NO synthase inhibitor L-NMMA. As shown in Figure 8, addition of L-NMMA had no effect on growth factor–induced SMC replication or its modulations by IFN-γ and TNF-α. This indicates that NO does not act by affecting the proliferative capacity of SMCs.

Both IFN-γ and TNF-α exert important effects on protein synthesis, which with time leads to differences in cell protein levels compared with control cells. This could result in substantial differences in cell mass per culture. However, the effects of IFN-γ+TNF-α treatment on nitrite and lactate accumulation were unchanged when calculated on a per cell protein basis (data not shown). This indicates that these effects were not secondary to changes in cell mass.

TABLE 3. Effects of Cytokines on Production of Nitrite and Lactate and Consumption of Glucose in Smooth Muscle Cells

Treatments	Nitrite (μM)	Lactate (mM)	Glucose (mM)	Lactate/glucose
Untreated	5.69±0.28	9.13±0.82	9.74±0.23	0.94
IFN-γ	5.66±0.49	5.78±0.45	8.89±0.28	0.65
TNF-α	8.35±0.81	8.66±0.28	10.26±0.06	0.84
IFN-γ+TNF-α	34.15±2.62*†	20.35±0.23*†	10.22±0.04	1.99*†
IFN-γ+TNF-α+L-NMMA	14.84±0.65	10.23±1.58	9.30±0.23	1.10

IFN-γ, interferon-γ; TNF-α, tumor necrosis factor-α; L-NMMA, N^G-monomethyl L-arginine. Values are mean±SEM (n=4).

Smooth muscle cells were exposed to cytokines (500 units/ml each), and media were analyzed for nitrite, lactate, and glucose after 48 hours.

*$p<0.01$ compared with untreated cells.

†$p<0.05$ compared with IFN-γ+TNF-α+L-NMMA–treated cells.

Discussion

NO is an important regulator of vascular tone. Endothelium-derived NO activates guanylate cyclase in SMCs and leads via a cGMP-dependent protein kinase to dephosphorylation of myosin light chains and muscle relaxation.[1–4] This represents a rapid paracrine mechanism for regulation of SMC contractility. In addition, SMCs can be stimulated to synthesize their own NO by cytokine stimulation.[16–18] We confirm and extend these observations by showing that cultured rat aortic SMCs produce nitric compounds from L-arginine in response to the cytokines, IFN-γ and TNF-α, and that this is due to a synergistic effect of the two cytokines, since neither of them caused significant nitrite production alone. Nitrite is only one of several end products of NO metabolism, and it can also be formed from other nitrosyl compounds. However, the majority of NO formed in a biological system is oxidized to nitrite,[26,27] and the accumulation of this compound parallels biological effects of NO such as vascular relaxation.[26] Therefore, it is reasonable to assume that the accumulation of nitrite in our cultures reflects the production of NO by the cells.

The main novel observation of the present study is that SMCs treated with IFN-γ and TNF-α exhibit an NO-dependent block in complexes I and II of the mitochondrial respiratory chain. This is accompanied by an increased lactate production, which indicates that glucose is metabolized by anaerobic glycolysis that is due to the inhibition of oxidative phosphorylation. The efficiency of energy production by anaerobic glycolysis is only a small fraction (approximately 10%) of that obtained in the aerobic part of glucose combustion. Therefore, it appears likely that SMCs exposed to the two cytokines would suffer from an insufficient energy supply, which would have a detrimental effect on the contractile capacity of the cells. Therefore, we suggest that NO may act on at least two levels to inhibit SMC contractility: a rapid cGMP-mediated effect and a more sluggish one on oxidative metabolism.

The mechanism by which NO blocks mitochondrial respiration is probably similar to the one elucidated in activated macrophages.[8–10] There, cytokine-induced NO forms nitrosyl–iron–sulfur complexes with several enzymes that contain nonheme iron in their prosthetic groups, such as aconitase in the citric acid cycle and complex I and complex II in the respiratory chain. Interestingly, nitrosyl–iron–sulfur complexes have recently been reported to exert endothelium-derived relaxing factor activity and were suggested to be stable forms of endothelium-derived relaxing factor.[28,29] The formation of such complexes could therefore prolong

TABLE 4. Viability of Vascular Smooth Muscle Cells Treated With Interferon-γ and Tumor Necrosis Factor-α

Doses (units/ml)	Viability of SMCs (%)		
	IFN-γ	TNF-α	IFN-γ+TNF-α
0	96.8±1.5	96.8±1.5	96.8±1.5
10	95.8±2.5	96.8±2.5	94.8±2.7
100	94.3±2.8	95.0±2.2	94.3±1.7
1,000	93.8±1.7	93.3±4.7	82.3±3.3*
3,000	92.5±2.4	90.0±2.6	75.3±4.6*

SMCs, smooth muscle cells; IFN-γ, interferon-γ; TNF-α, tumor necrosis factor-α. Values are mean±SEM (n=4).

Cells were exposed to cytokines at different doses for 72 hours, and viability was assayed by trypan blue exclusion.

*Significantly different from control cells at $p<0.05$.

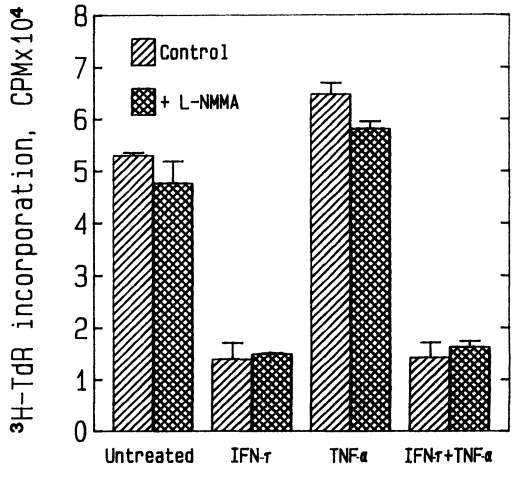

FIGURE 8. *Bar graph showing that smooth muscle cell replication in cytokine-treated cultures is not dependent on nitric oxide. L-NMMA, N^G-monomethyl L-arginine; TdR, thymidine; IFN-γ, interferon-γ; TNF-α, tumor necrosis factor-α. Quiescent cells were stimulated to enter the cell cycle by addition of 10% fetal calf serum with or without IFN-γ and/or TNF-α (100 units/ml each). DNA replication was determined as described in "Materials and Methods." L-NMMA (100 μM) was added to some of the cultures. Bars indicate mean±SEM* (n=3).

the effects of NO and may explain the slow kinetics of the metabolic changes that we have studied. It appears likely that such NO-induced enzyme inhibition is reversible, since reconstitution of the iron–sulfur complex restores aconitase activity in NO-exposed tumor cells.[30]

It is possible that the sensitivity to an inhibited mitochondrial respiration may differ between the various energy-dependent processes in the cell. For example, membrane ionic pumps of SMCs preferentially use ATP derived from anaerobic glycolysis, even under fully aerobic conditions.[31–33] The NO-associated increase in anaerobic glycolysis may therefore supply ionic pumps with large amounts of ATP while there is a shortage of ATP for contraction in the NO-producing cell. This may be important for cell survival, which is critically dependent on the function of the membrane pumps. Interestingly, the effects of NO on energy metabolism differ in this respect from those of H_2O_2 and free radicals such as the superoxide anion, which have been shown to inhibit both glycolysis and mitochondrial respiration during cytotoxic attacks.[34,35]

The sequence of events that leads from cytokine stimulation to NO production is still unclear. Its dependence on protein synthesis suggests that it involves synthesis of the enzyme NO synthase, but it is also possible that the protein(s) involved represents auxilliary factors in the NO pathway. Further studies are obviously needed to clarify whether IFN-γ and TNF-α induce expression of the NO synthase gene or affect the level of NO synthase mRNA, the synthesis of NO synthase protein, the activation of the enzyme, or the transport of substrate or cofactors into the NO-synthesizing compartment in the cell.

The in vivo regulation of NO production may differ between phenotypic forms of SMCs. Whereas SMCs of the arterial media exhibit a contractile phenotype with an abundance of microfilaments, proliferating SMCs of regenerating arteries express the "synthetic" phenotype that is dominated by protein synthetic organelles.[36] Our SMC cultures can be considered to represent the synthetic phenotype; therefore, it remains to be determined whether contractile SMCs of the arterial media respond to cytokines by autocrine NO production and inhibition of the respiratory chain.

The capacity for autocrine NO production in SMCs could be important both physiologically and pathophysiologically, since SMCs are the most abundant cells of the blood vessels and directly control vascular tone, systemic blood pressure, and regional blood flow. Hyperemia is a characteristic component of inflammatory conditions and is considered necessary for delivery of both humoral and cellular components of the immune system to the site of inflammation. IFN-γ is produced by one of the major cell types of the immune system, the T lymphocyte, and TNF is produced both by macrophages (TNF-α) and T lymphocytes (TNF-β, also called lymphotoxin).[37,38] Both TNF forms act on the same receptor,[38] and TNF-α, TNF-β, and IFN-γ are known to be released at inflammatory foci.[37,38] This would be expected to cause vasodilation by inducing SMC synthesis of NO, which, in turn, may contribute to the hyperemia of inflammation.

Vascular NO production may also be important in pathological conditions. For example, there is good evidence for local cytokine production in inflammatory diseases such as rheumatoid arthritis,[39] which is also characterized by local hyperemia of the afflicted joints. In atherosclerosis, both IFN-γ and TNF are known to be produced in the plaque,[40,41] which also contains large amounts of "synthetic" SMCs.[36] Here, a cytokine-induced NO synthesis may compensate for the loss of endothelial function and the attenuated endothelium-derived vascular relaxation[42,43] and participate in the regulation of vascular tone as well as SMC proliferation.[44] Cytokine-induced NO-mediated vasodilation could also be involved in the pathogenesis of septic shock.[45,46] Finally, it has been proposed that increased NO production may trigger acute metabolic failure and subsequent tissue damage in patients with respiratory chain defects,[47] e.g., the acute onset of blindness in Leber's hereditary optic neuropathy and the strokelike episodes in the MELAS syndrome (Myopathy, Encephalopathy, Lactic Acidosis, and Strokelike episodes). The demonstration that autocrine NO production can inhibit the respiratory chain in SMCs lends support to a possible pathophysiological role for NO in mitochondrial disorders.

In conclusion, the present data show that IFN-γ and TNF-α act synergistically to induce NO production in SMCs. This is accompanied by a block in mitochondrial respiration, and the energy production is essentially reduced to anaerobic glycolysis. These findings underline the biological importance of NO as a regulator of cellular metabolism and demonstrate an additional level of action that could be important for the contractile capacity of vascular SMCs.

Acknowledgments

We thank Marianne Almqvist, Ylva Nyberg, Stina Nygren, and Qi Wu for their skillful technical assistance and Dr. Åke Wennmalm for constructive comments on the manuscript.

References

1. Vane JR, Änggård EE, Botting RM: Regulatory functions of the vascular endothelium. *N Engl J Med* 1990;323:27–36
2. Moncada S, Higgs EA, Hodson HF, Knowles RG, Lopez-Jaramillo P, McCall T, Palmer RM, Radomski MW, Rees DD, Schulz R: The L-arginine:nitric oxide pathway. *J Cardiovasc Pharmacol* 1991;17(suppl):S1–S9
3. Palmer RMJ, Ferrige AG, Moncada S: Nitric oxide release accounts for the biological activity of endothelium-derived relaxing factor. *Nature* 1987;327:524–526
4. Ignarro LJ, Bug GM, Wood KS, Byrns RE: Endothelium-derived relaxing factor produced and released from artery and vein is nitric oxide. *Proc Natl Acad Sci U S A* 1987;84:9265–9269
5. Bredt DS, Snyder SH: Isolation of nitric oxide synthase: A calmodulin-requiring enzyme. *Proc Natl Acad Sci U S A* 1990;87:682–685
6. Schmidt HHHW, Pollock JS, Nakane M, Gorsky LD, Förstermann U, Murad F: Purification of a soluble isoform of guanylyl cyclase-activating factor synthase. *Proc Natl Acad Sci U S A* 1991;88:365–369
7. Bredt DS, Hwang HW, Glatt CE, Lowenstein C, Reed RR, Snyder SH: Cloned and expressed nitric oxide synthase structurally resembles cytochome P-450 reductase. *Nature* 1991;351:714–718
8. Stuehr DJ, Cho HJ, Kown NS, Weise MF, Nathan CF: Purification and characterization of the cytokine-induced macrophage nitric oxide synthase: An FAD- and FMN-containing flavoprotein. *Proc Natl Acad Sci U S A* 1991;88:7773–7777
9. Drapier J-C, Hibbs JB: Differentiation of murine macrophages to express nonspecific cytotoxicity for tumor cells results in L-arginine-dependent inhibition of mitochondrial iron-sulfur enzymes in the macrophage effector cells. *J Immunol* 1988;140:2829–2838
10. Drapier J-C, Pellat C, Henry Y: Generation of EPR-detectable nitrosyl-iron complexes in tumor target cells cocultured with activated macrophages. *J Biol Chem* 1991;266:10162–10167

11. Stuehr DJ, Nathan CF: Nitric oxide: A macrophage product responsible for cytostasis and respiratory inhibition in tumor target cells. *J Exp Med* 1989;169:1543–1555
12. Gross SS, Stuehr DJ, Aisaka K, Jaffe EA, Levi R, Griffith OW: Macrophage and endothelial cell nitric oxide synthesis: Cell type selective inhibition by N^G-nitroarginine and N^G-methylarginine. *Biochem Biophys Res Commun* 1990;170:96–103
13. Pollock J, Förstermann U, Mitchell JA, Warner TD, Schidt HHHW, Nakane M, Murad F: Purification and characterization of particulate endothelium-derived relaxing factor synthase from cultured and native bovine aortic endothelial cells. *Proc Natl Acad Sci U S A* 1991;88:10480–10484
14. Xie Q, Cho HJ, Calaycay J, Mumford RA, Swiderek KM, Lee TD, Ding A, Troso T, Nathan C: Cloning and characterization of inducible nitric oxide synthase from mouse macrophages. *Science* 1992; 256:225–228
15. Lyons CR, Orloff GJ, Cunningham JM: Molecular cloning and functional expression of an inducible nitric oxide synthase from a murine macrophage cell line. *J Biol Chem* 1992;267:6370–6374
16. Busse R, Mülsch A: Induction of nitric oxide synthase by cytokines in vascular smooth muscle cells. *FEBS Lett* 1990;275:87–90
17. Wood KS, Buga GM, Byrns RE, Ignarro LJ: Vascular smooth muscle-derived relaxing factor (MDRF) and its close similarity to nitric oxide. *Biochem Biophys Res Commun* 1990;170:80–88
18. Beasley D, Schwartz JH, Brenner BM: Interleukin 1 induces prolonged L-arginine-dependent cyclic guanosine monophosphate and nitrite production in rat vascular smooth muscle cells. *J Clin Invest* 1991;178:823–829
19. Ding AH, Nathan CF, Stuehr DJ: Release of reactive nitrogen intermediates and reactive oxygen intermediate from mouse peritoneal macrophages. *J Immunol* 1988;141:2407–2414
20. Hansson GK, Jonasson L, Holm J, Clowes MM, Clowes AW: γ-Interferon regulates vascular smooth muscle proliferation and Ia antigen expression in vivo and in vitro. *Circ Res* 1988;63:712–719
21. Hansson GK, Hellstrand M, Rymo L, Rubbia L, Gabbiani G: Interferon-γ inhibits proliferation and expression of differentiation-specific α-smooth muscle actin in arterial smooth muscle cells. *J Exp Med* 1989;170:1595–1608
22. Hansson GK, Schwartz SM: Evidence for cell death in the vascular endothelium in vivo and in vitro. *Am J Pathol* 1983;112:278–286
23. Lundholm L: Lactic acid assay with L(+) lactic acid dehydrogenase from rabbit muscle. *Acta Physiol Scand* 1963;58:243–249
24. Trinder P: Determination of blood glucose using an oxidase-peroxidase system with a non-carcinogenic chromogen. *J Clin Pathol* 1969;22:158–161
25. Granger DL, Lehninger AL: Sites of inhibition of mitochondrial electron transport in macrophage-injured neoplastic cells. *J Cell Biol* 1982;95:527–535
26. Furchgott R, Jothianandan D, Frey AD: Endothelium-derived relaxing factor: Some old and new findings, in Moncada S, Higgs E (eds): *Nitric Oxide From L-Arginine: A Bioregulatory System.* Amsterdam, Elsevier Science Publishing Co, Inc, 1990, pp 5–17
27. Murphy ME, Sies H: Reversible conversion of nitroxyl anion to nitric oxide by superoxide dismutase. *Proc Natl Acad Sci U S A* 1991;88:10860–10864
28. Vanin A: Hypothesis: Endothelium-derived relaxing factor is a nitrosyl iron complex with thiol ligands. *FEBS Lett* 1991;289:1–3
29. Mülsch A, Mordvintcev P, Vanin AF, Busse R: The potent vasodilating and guanylyl cyclase activating dinitrosyl-iron (II) complex is stored in a protein-bound form in vascular tissue and is released by thiols. *FEBS Lett* 1991;294:252–256
30. Drapier J-C, Hibbs JB: Murine cytotoxic activated macrophages inhibit aconitase in tumor cells. *J Clin Invest* 1986;78:790–797
31. Paul RJ, Baner M, Pease W: Vascular smooth muscle aerobic glycolysis linked to sodium and potassium transport processes. *Science* 1979;206:1414–1416
32. Kutchain H, Geddis LM: Regulation of glycolysis in rat aorta. *Am J Physiol* 1984;247(*Cell Physiol* 16):C107–C114
33. Paul RJ: Smooth muscle energetics. *Annu Rev Physiol* 1989;51: 331–349
34. Hyslop PA, Hinshaw DB, Halsey WA Jr, Schraufstätter IU, Sauerheber RD, Spragg RG, Jackson JH, Cochrane CG: Mechanisms of oxidant-mediated cell injury: The glycolytic and mitochondrial pathways of ADP phosphorylation are major intracellular targets inactivated by hydrogen peroxide. *J Biol Chem* 1988;263:1665–1675
35. Correti MC, Koretsune Y, Kusuoka H, Chacko VP, Zweier JL, Marban E: Glycolytic inhibition and calcium overload as consequences of exogenously generated free radicals in rabbit hearts. *J Clin Invest* 1991;88:1014–1025
36. Thyberg J, Hedin U, Sjölund M, Palmberg L, Bottger BA: Regulation of differentiated properties and proliferation of arterial smooth muscle cells. *Arteriosclerosis* 1990;10:966–990
37. Trinchieri G, Perussia B: Immune interferon: A pleiotropic lymphokine with multiple effects. *Immunol Today* 1985;6:131–133
38. Beutler B, Cerami A: The biology of cachectin/TNFα: A primary mediator of the host response. *Annu Rev Immunol* 1989;7:625–655
39. Klareskog L, Rönnelid J, Gudmundsson S, Karlsson-Parra A: Rheumatoid arthritis. *Curr Opin Immunol* 1991;3:912–916
40. Hansson GK, Holm J, Jonasson L: Detection of activated T lymphocytes in the human atherosclerotic plaque. *Am J Pathol* 1989; 135:169–175
41. Barath P, Fishbein MC, Cao J, Berenson J, Helfant RH, Forrester JS: Detection and localization of tumor necrosis factor in human atheroma. *Am J Cardiol* 1990;65:297–303
42. Chester AH, O'Neil GS, Moncada S, Tadjkarimi S, Yacoub MH: Low basal and stimulated release of nitric oxide in atherosclerotic epicardial coronary arteries. *Lancet* 1990;336:897–900
43. Zeiher AM, Drexler H, Wollschläger HM, Just H: Modulation of coronary vasomotor tone in humans: Progressive endothelial dysfunction with different early stages of coronary atherosclerosis. *Circulation* 1991;83:391–401
44. Garg UC, Hasid A: Nitric oxide-generating vasodilators and 8-bromocyclic guanosine monophosphate inhibit mitogenesis and proliferation of cultured rat vascular smooth muscle cells. *J Clin Invest* 1989;83:1774–1777
45. Kilbourn RG, Gross SS, Jubran A, Adams J, Griffith OW, Levi R, Lodato RF: N^G-Methyl-L-arginine inhibits tumor necrosis factor-induced hypotension: Implications for the involvement of nitric oxide. *Proc Natl Acad Sci U S A* 1990;87:3629–3632
46. Petros A, Bennett D, Vallance P: Effect of nitric synthase inhibitors on hypotension in patients with septic shock. *Lancet* 1991;338: 1557–1558
47. Larsson N-G, Andersen O, Holme E, Oldfors A, Wahlström J: Leber's hereditary optic neuropathy and complex one deficiency in muscle. *Ann Neurol* 1991;30:701–708

Interferon-gamma and tumor necrosis factor synergize to induce nitric oxide production and inhibit mitochondrial respiration in vascular smooth muscle cells.

Y Geng, G K Hansson and E Holme

Circ Res. 1992;71:1268-1276
doi: 10.1161/01.RES.71.5.1268
Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231

Print ISSN: 0009-7330. Online ISSN: 1524-4571