Interleukin 1 Activates Soluble Guanylate Cyclase in Human Vascular Smooth Muscle Cells through a Novel Nitric Oxide–independent Pathway

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Summary

Recent demonstration of cytokine-inducible production of nitric oxide (NO) in vascular smooth muscle cells (VSMC) from rat aorta has implicated VSMC-derived NO as a key mediator of hypotension in septic shock. Our studies to determine whether an inducible NO pathway exists in human VSMC have revealed a novel cytokine-inducible, NO-independent pathway of guanylate cyclase activation in VSMC from human saphenous vein (HSVSMC). Interleukin 1 (IL-1), tumor necrosis factor (TNF), interferon γ (IFN-γ) and Escherichia coli lipopolysaccharide (LPS) increased cGMP at 24 h, whereas IL-2 and IL-6 were ineffective. The effect of IL-1 on cyclic guanosine 3′,5′-monophosphate (cGMP) was delayed, occurring after 6 h of exposure, and was maximal after 10 h. Methylene blue and LY83583 reversed the IL-1-induced increase in cGMP, suggesting that it was mediated by activation of soluble guanylate cyclase. However, IL-1-induced cGMP in HSVSMC was not inhibited by extracellular hemoglobin. Also, the effect of IL-1 on cGMP was not reversed by nitro- or methyl-substituted L-arginine analogs, aminoguanidine, or diphenyleneiodonium, all of which inhibit IL-1-induced NO synthase in rat aortic VSMC (RAVSMC). IL-1-induced cGMP in HSVSMC was also independent of tetrahydrobiopterin and extracellular L-arginine, as it was not affected by 2,4-diamino-6-hydroxyprytinimide, an inhibitor of tetrahydrobiopterin biosynthesis, and was similar in L-arginine-free and L-arginine-containing media. Analysis of NO synthase mRNA with the use of polymerase chain reaction indicates that levels of mRNA for inducible NO synthase are several orders of magnitude lower in IL-1-treated human HSVSMC than in IL-1-treated RAVSMC. IL-1-induced cGMP was also NO independent in human umbilical artery VSMC, and NO dependent in rat vena cava VSMC. Together these results indicate that IL-1 activates a novel NO-independent pathway of soluble guanylate cyclase activation in human VSMC.

Recent evidence has implicated the free radical, nitric oxide (NO)1, as a key physiological and pathophysiological regulator of vascular tone. NO is produced enzymatically from L-arginine by a family of enzymes termed NO synthases (1). Activation of a calcium-calmodulin–dependent, constitutive NO synthase within the endothelium accounts for the action of endothelium-dependent vasodilators in animal (2, 3) and human blood vessels (4, 5), and contributes to vasodilation in normal animals and humans (4, 6). Studies with rodent cells and tissues have implicated a cytokine-inducible NO synthase as a mediator of the pathophysiological vasodilation associated with septic shock. IL-1, TNF, and bacterial LPS induce NO synthase activity in rat aortic vascular smooth muscle cells (RAVSMC; 7–9), which is demonstrable as increased production of cyclic guanosine 3′,5′-monophosphate (cGMP) and nitrite. NO-induced cGMP production is responsible for diminished vascular contraction observed in intact rat aorta treated with IL-1 in vitro (10, 11). To establish whether VSMC–derived NO may contribute to cytokine and LPS-induced hypotension in humans, we sought to determine whether a similar cytokine-inducible NO pathway exists in human VSMC.

In this study, we demonstrate that IL-1, TNF, IFN-γ, and LPS induce a novel pathway of guanylate cyclase activation in VSMC from human saphenous vein (HSVSMC). This pathway is distinct from the NO synthase pathway activated in RAVSMC in that it is not inhibited by NO synthase inhibitors including nitro- and methyl-substituted L-arginine.
Materials and Methods

Culture of VSMC. Human VSMC were grown by explant technique from unused portions of saphenous veins harvested for coronary artery bypass surgery (New England Medical Center) as previously described (12,13), or were obtained from Dr. Peter Libby (Brigham and Women's Hospital, Boston, MA). VSMC were also grown from human umbilical arteries. Umbilical cords were dissected free from the cord, cleaned of adventitia and endothelium, and VSMC grown from explants of the media. Cells were passaged every 7-30 d by harvesting with trypsin-EDTA and seeding at a 1:3 ratio. For experiments, cells were grown in DMEM, supplemented with 10-20% FCS, glutamine, penicillin, and streptomycin, and used between passages two and six after reaching confluence at 7-30 d.

VSMC were isolated from rat aorta and rat vena cava by enzymatic dissociation using standard methods as described previously (7). VSMC were grown in DMEM containing 10% FCS, glutamine, and antibiotics, passed once a week by harvesting with trypsin-EDTA, and plated at a 1:5 ratio. VSMC were used between passages 3 and 12 after reaching confluence at 4-7 d.

VSMC Incubations. HSVSMC were washed three times with HBSS and incubated with or without cytokines or LPS in DMEM containing 5% FCS (DMEM-5) for 6 or 24 h. The cytokines used in this study included human rIL-1 (Cistron Biotechnology, Inc., Pine Brook, NJ), human rIFN-α (Genzyme Corp., Boston, MA), human IL-2 (Endogen, Inc., Boston, MA), human rIL-6 (Genzyme Corp.), human rIFN-γ (Genentech, South San Francisco, CA), and E. coli LPS (serotype 055:B5; Sigma Chemical Co., St. Louis, MO). RAVSMC were incubated with or without IL-1 under identical conditions for 24 h. The phosphodiesterase inhibitor, isobutyryl-methylxanthine (IBMX; 0.1 or 1 mM) was added to the cells 15-30 min before obtaining cell extracts for cGMP determination. Inhibitors of NO synthesis/action were added either at the beginning of the incubation with IL-1 or towards the end, before the addition of IBMX.

In most experiments, the L-arginine concentration of the tissue culture media (DMEM) was 400 μM. For L-arginine depletion studies, cells were incubated in MEM with or without L-arginine (600 μM; Select-Amine-kit; GIBCO BRL, Gaithersburg, MD) for 48 h before activation with IL-1. HSVSMC were then activated with IL-1 in fresh media with or without L-arginine. The effect of NO synthase inhibitors was tested in media containing either no (0 μM) or low (50 μM) L-arginine. HSVSMC were incubated for 6 h with or without IL-1 in MEM containing 50 μM L-arginine, and inhibitors were added directly to this media at the end of the incubation period. Alternatively, VSMC or HSVSMC were incubated for 24 h with or without IL-1 in DMEM-5 containing 400 μM L-arginine. 40 min before the end of this incubation period, the cells were washed and the media replaced with Earle's BSS (L-arginine free) with or without NO synthase inhibitors.

Cyclic GMP Measurements. cGMP was extracted from the cells by rapid aspiration of the medium and addition of ice-cold 0.1 N HCl to each well. Cells were incubated 1 h on ice and the extracts frozen at -20°C before assay for cGMP. The cGMP content of cell extracts was determined by RIA using a commercial kit (Advanced Magnetics, Inc., Cambridge, MA).

Nitrite Assay. Nitrite was measured by a standard method (14) in which aliquots of cell supernatant were mixed with an equal volume of Greiss reagent (0.05% naphthylenediamine dihydrochloride, 0.5% sulfanilamide, and 2.5% phosphoric acid) and incubated at room temperature for 10 min. The absorbance at 550 nm was measured, and nitrite concentration determined using sodium nitrite as a standard and distilled water as a blank. The background nitrite values of each media was determined and subtracted from experimental values.

RT-PCR. Total RNA was isolated by the acid guanidinium thiocyanate-phenol-chloroform method (15). 2 μg RNA was reversed transcribed for 1 h at 37°C with 200 U of Superscript Moloney murine leukemia virus reverse transcriptase (GIBCO BRL), using an oligo(dT) primer and the buffer supplied by the manufacturer, in a total volume of 20 μl. The reaction was terminated by heating to 95°C for 5 min, and 2.5 μl of this first strand cDNA was added to each PCR reaction. PCR mixes included 50 mM KCl, 10 mM Tris, pH 8.3, 1.0 mM MgCl₂, 0.01 mg/ml gelatin, 200 μM of each deoxynucleotide triphosphate, 250 nM of each primer, and 0.5 U Taq DNA polymerase (Pharmacia, Piscataway, NJ) in a total volume of 25 μl, overlaid with 25 μl mineral oil. The inducible NO synthase PCR primers (TAGAG-GAACATCTGCGCAAGG and TGGCCGACCTGATGTTGCCA) were homologous to inducible human hepatocyte NO synthase (16) and to rat vascular smooth muscle NO synthase (17). Amplification was performed as follows: 94°C for 2 min (initial melt); 26-40 cycles of 94°C for 1 min, 57°C for 2 min, 72°C for 2 min; and then 72°C for 5 min (final extension). Products were size separated by electrophoresis in either a 2% agarose or 5% polyacrylamide gel and visualized by ethidium bromide staining. In some cases, PCR bands were quantitated by adding 0.25 μCi (33 nM) [32P]dCTP to the PCR reaction, cutting the PCR band from the gel and counting in a liquid scintillation counter.

Drugs. Human rIL-1 was a gift of Dr. Richard Dondoro (Cistron Biotechnology, Inc.). DPI was a gift of Dr. Owen T. G. Jones (University of Bristol, Bristol, UK). Human hemoglobin was a gift of Dr. Robert Valeri (Naval Blood Research Laboratory, Boston, MA), and contained 97.2% oxyhemoglobin and 2.8% methemoglobin. IBMX, N⁶-nitro-L-arginine methyl ester (L-NAME), N⁶-nitro-L-arginine (L-NAME), aminoguanidine bicarbonate, and 2,4-diamino-6-hydroxypropyrimidine were obtained from Sigma.

Figure 1. IL-1 induces a delayed increase in cGMP accumulation in HSVSMC. HSVSMC were incubated with IL-1 (200 U/ml) in DMEM-5 and cGMP determined after 1-24 h. IBMX (0.1 mM) was added 30 min before cGMP determination. n = 4 replicates. Error bars which are not visible are less than the width of the symbol.
Chemical Co. Nω-monomethyl-Arg (L-NMMA) acetate and L-NMMA p-hydroxyazobenzene-p'-sulfonate salt were obtained from Calbiochem-Novabiochem Corp. (La Jolla, CA). (6R)-5,6,7,8-tetrahydrobiopterin dihydrochloride was from ICN Biomedicals (Irvine, CA).

Data Analysis. Values are expressed as mean ± SE. Significant differences were determined by unpaired Student’s t test, and p < 0.05 was considered statistically significant.

Results

IL-1, TNF, LPS, and IFN-γ Increase cGMP in HSVSMC.
IL-1 induced a delayed increase in cGMP in HSVSMC. cGMP was significantly increased after 6 h of exposure to IL-1 (p <0.0001), continued to increase reaching maximal levels after 10 h of exposure, and then decreased towards control levels (Fig. 1). The magnitude of the IL-1 effect was similar whether cells were studied in the presence or absence of a phosphodiesterase inhibitor, IBMX (Fig. 2). Whereas IBMX increased basal levels of cGMP fivefold, a 6-h exposure to IL-1 increased GMP fourfold in the absence of IBMX, and 4.5-fold in the presence of IBMX. Thus, IL-1–induced changes in cGMP reflect increased production rather than decreased degradation by phosphodiesterase. All other experiments were conducted with IBMX.

HSVSMC incubated 24 h with TNF (1 nM), IFN-γ (10 nM), or E. coli LPS (100 μg/ml), showed an increase in cGMP similar to that of IL-1 (1 nM). In contrast, IL-2 (10 nM) and IL-6 (10 nM) did not affect cGMP production (Fig. 3).

Methylene Blue and LY83,583 Reverse IL-1–induced cGMP in HSVSMC.
IL-1–induced cGMP accumulation was reversed by two soluble guanylate cyclase inhibitors, methylene blue and LY 83,583 (Fig. 4 A). Both methylene blue (10 μM) and LY 83,583 (10 μM) lowered cGMP content in nontreated HSVSMC, and decreased the IL-1–induced increase in cGMP by 90 and 91% (p <0.002). In contrast, extracellular hemoglobin (30 μM) did not significantly affect cGMP levels in control or IL-treated HSVSMC (Fig. 4 B).

l-Arginine Analogs Do Not Reverse IL-1–induced cGMP in HSVSMC.
IL-1–induced cGMP production was not attenuated by l-arginine analogs containing methyl or nitro substitutions of the guanidino nitrogen. Neither l-NMMA, l-NAME, l-NoArg, nor aminoguanidine inhibited IL-1–induced increases in cGMP.

Figure 2. IL-1 increase cGMP in the presence and absence of a phosphodiesterase inhibitor, IBMX. HSVSMC were incubated with IL-1 (200 U/ml) and cGMP determined after 6 h. IBMX (1 mM) was added to some of the wells 20 min before cGMP determination. n = 4 replicates.

Figure 3. Effect of cytokines on cGMP accumulation in HSVSMC. HSVSMC were incubated with IL-1 (1 nM or 170 U/ml), TNF (1 nM), E. coli LPS (100 μg/ml), IFN-γ (10 nM), IL-2 (10 nM), or IL-6 (10 nM) for 24 h. IBMX (0.1 mM) was added 30 min before cGMP determination. n = 4 replicates. (* p < 0.02, significantly different from control HSVSMC.)

Figure 4. IL-1–induced increases in cGMP in HSVSMC are attenuated by methylene blue (MB) and LY83583 (LY), but not by hemoglobin (Hb). HSVSMC were incubated with IL-1 (200 U/ml) for 6 h. MB or LY (each 10 μM; A) or Hb (30 μM; B) were added 40 min before, and IBMX 20 min before cGMP determination. n = 4 replicates. (+) p < 0.01, significantly different from control HSVSMC; (* p < 0.002, significantly different from IL-1–treated HSVSMC.)
duced cGMP in HSVSMC, when tested at concentrations of 1 mM in the presence of 50 µM t-arginine (Fig. 5). Likewise, neither L-NoArg nor L-NMMA (0.03–1 mM) reversed the increased cGMP accumulation in IL-1−treated human VSMC, when tested in balanced salt solution that contained no L-arginine (Fig. 6). Under similar experimental conditions, aminoguanidine (0.01–3 mM) was equally ineffective at reversing IL-1−induced cGMP in HSVSMC (Fig. 7), as were t-NAME and l-NMMA p-hydroxyazobenzene-p’-sulfonate salt (data not shown). In contrast, L-NMMA (Fig. 6) and aminoguanidine (1–1,000 µM; Fig. 7) were potent inhibitors of IL-1−induced cGMP in RAVSMC under identical experimental conditions.

**DPI Does Not Reverse IL-1−induced cGMP in HSVSMC.**

The flavoprotein inhibitor, DPI (0.3–3 µM) attenuated the IL-1−induced increase in cGMP in RAVSMC in a concentration-dependent fashion (Fig. 8 A). These concentrations of DPI did not affect the increase in cGMP caused by sodium nitroprusside in these cells (data not shown). In contrast, DPI (1–3 µM) did not significantly affect IL-1−induced cGMP in HSVSMC (Fig. 8 B).

**IL-1−induced cGMP in HSVSMC Is Independent of Extracellular L-Arginine.**

IL-1−increased cGMP was not attenuated in HSVSMC that had been cultured for 48 h in L-arginine−free MEM, then stimulated for 6 h with IL-1 in fresh L-arginine−free MEM (Fig. 9). Also, repletion of L-arginine in some of the L-arginine−depleted VSMC 1 h before cGMP determination did not affect the response to IL-1.

**IL-1−induced cGMP in HSVSMC Is Independent of Tetrahydrobiopterin.**

2,4-diamino-6-hydroxypyrimidine (DAHP) is an inhibitor of tetrahydrobiopterin synthesis, which inhibits NO synthase activity in several cell types by depleting intracellular levels of this cofactor. Addition of DAHP (2.5–10 mM) at the beginning of the 24-h incubation with IL-1 block IL-1−induced cGMP in RAVSMC in a concentration−dependent manner (Fig. 10 A). The effect of DAHP (10 mM) was reversed by the addition of 100 µM tetrahydrobiopterin to the media 80 min before cGMP determination, supporting the hypothesis that the inhibitory effect of DAHP was due to depletion of tetrahydrobiopterin. In contrast, DAHP and tetrahydrobiopterin had no effect on IL-1−induced cGMP in HSVSMC, when added under the same conditions (Fig. 10 B).
**Extracellular Nitrite Accumulation.** Whereas nitrite was detectable in supernatants of HSVSMC, treatment with IL-1 did not significantly affect nitrite accumulation. The nitrite concentration of supernatants from control and IL-1-treated HSVSMC were 2.0 ± 0.2 μM and 2.5 ± 0.3 μM, respectively, at 24 h, and 4.5 ± 1.1 μM and 3.5 ± 0.3 μM at 48 h.

**Detection of NO Synthase mRNA by RT-PCR.** cDNA prepared from rat and human VSMC was amplified using a single set of primers that had complete homology to both rat and human inducible NO synthase (16, 17). Amplification of cDNA prepared from RAVSMC, which had been incubated with IL-1 for 24 h, revealed a PCR product of the expected size (255 bp) that was detected after as few as 20 cycles (data not shown). Amplification of cDNA from IL-1–treated rat VSMC showed an exponential increase in PCR product between 20 and 26 cycles; no further increase in PCR product was seen with 28–30 cycles of amplification (data not shown). Thus, 25 or 26 amplification cycles were used for further studies. Exposure of RAVSMC to IL-1 produced a time-dependent increase in NO synthase mRNA, that was maximal at 4 and 24 h (Fig. 11 A). When cDNA from IL-1–treated RAVSMC was amplified for 25 cycles in the presence of [32P]dCTP, an intense band was visible by ethidium bromide staining of products (Fig. 11 B), whereas control VSMC gave only a faint band. Quantitation of the 32P content of the bands shown in Fig. 11 B by liquid scintillation counting revealed that IL-1 treatment of rat VSMC produced an 18-fold increase in NO synthase mRNA levels (control VSMC, 2,702 cpm; 24-h IL-1–treated VSMC, 49,455 cpm). In contrast, cDNA prepared from HSVSMC treated with IL-1 for 0–24 h showed no visible PCR products by either ethidium bromide staining (Fig. 11 B), or by liquid scintillation counting (less than the background level of 228 cpm). When cDNA from HSVSMC was amplified further, for 30–32 cycles, faint bands of the expected size were visible in IL-1–treated samples.

**Cytokine-induced cGMP in Human Umbilical Artery and Rat Vena Cava VSMC.** To determine whether the difference in mechanism of IL-1 action in rat aorta and human saphenous VSMC reflects differences between arteries and veins, or between species, we studied IL-1–induced cGMP in VSMC from human umbilical artery and rat vena cava. In human umbilical artery VSMC, cGMP was not significantly increased by IL-1 alone (20 ng/ml), whereas treatment with IL-1 (20 ng/ml) and TNF (50 ng/ml) for 6 h resulted in a 44–130% increase in cGMP (data not shown). cGMP was no longer increased at 24 h, suggesting a more rapid time course, similar to the time course in HSVSMC. Nitrite was not detectable in 24-h supernatants obtained from either control or IL-1- and TNF-treated human umbilical artery VSMC. Finally, the t-arginine analog l-NMMA did not affect cGMP in human umbilical artery VSMC stimulated with IL-1 and TNF (Fig. 12 A).

In rat vena cava VSMC, cGMP was significantly increased after 3-h incubation with IL-1 (20 ng/ml), and continued to increase up to 22-fold at 24 h (data not shown). Nitrite was detectable in 24-h supernatants from control cells, and...
Discussion

A rapidly expanding literature has implicated a role for cytokine-inducible NO synthesis in the pathogenesis of septic shock. Cytokines induce NO synthase activity in VSMC, suggesting that this intracellular source of NO may play a major role in vascular relaxation during sepsis. In RAVSMC, NO synthase activity is detectable by prolonged, L-arginine-dependent increases in cGMP production and accumulation of nitrite in the media (7), and is associated with an increase in inducible NO synthase mRNA (18). In this study, we report that IL-1 induces a similar delayed and prolonged increase in cGMP production in VSMC cultured from human saphenous vein. As in RAVSMC, TNF and E. coli LPS also increased cGMP in human VSMC. IFN-γ, which is ineffective at inducing NO synthase in RAVSMC (18, 19), also increased cGMP in HSVSMC. Thus, inflammatory cytokines increase the vasodilatory mediator cGMP in both RAVSMC and HSVSMC. However, the mechanism of IL-1 action was distinct in human VSMC. First, the time course of IL-1 action differed. cGMP increases after a lag period of >3 h in both cell types, however the maximal response occurs earlier in HSVSMC than in RAVSMC (10 versus 36 h; 7 and Fig. 1). Second, studies with inhibitors indicated that the mechanism of IL-1 action in HSVSMC involves activation of soluble guanylate cyclase but does not involve NO.

t-arginine analogs that contain methyl or nitro substitutions on the guanido nitrogen are competitive inhibitors of NO synthase (2, 20, 21), which consistently inhibit all constitutive and inducible NO syntheses described to date in various cell types. Neither monomethyl- nor nitro-substituted t-arginine analogs attenuated IL-1-induced increases in cGMP in human VSMC when tested at concentrations ≤1 mM either in the absence of t-arginine or in the presence of 50 μM L-arginine. In fact, there was a tendency for t-arginine analogs to increase IL-1-induced cGMP in HSVSMC, suggesting that NO produced by the cells may attenuate the IL-
1-induced response. In contrast, all l-arginine analogs tested inhibit cytokine-induced NO production in RAVSMC, under similar experimental conditions. Aminoguanidine is a nucleophilic hydrazine compound that contains two equivalent guanidino nitrogens, and has been shown to inhibit cytokine-inducible NO synthase in pancreatic β cells (22), presumably by acting as a competitive inhibitor of NO synthase. Our results indicate that aminoguanidine is also a potent inhibitor of cytokine-inducible NO synthase in RAVSMC, and is effective at concentrations as low as 1 μM. In contrast, IL-1-induced cGMP in HSVSMC was not affected by aminoguanidine.

Purified constitutive and inducible NO synthases are NADPH dependent (23) and contain flavin adenine dinucleotide and flavin mononucleotide (24, 25), which act as cofactors for NO synthase (26). Both macrophage and endothelial NO synthases are inhibited by DPI (27), an inhibitor of NADPH-dependent flavoproteins (28, 29). Our results indicate that cytokine-inducible NO synthase in RAVSMC is also inhibitable by DPI, whereas IL-1-induced cGMP in HSVSMC was not affected. These results provide additional evidence that the increase in cGMP in HSVSMC does not involve NO synthase or a related NADPH-dependent flavoprotein.

Purified constitutive and inducible NO synthases also contain tetrahydrobiopterin (30, 31) which is an essential cofactor for NO synthase oxidation of l-arginine to citrulline and NO (30). Cytokine-inducible NO synthase in endothelial cells (32, 33), VSMC (19), macrophages (34), and fibroblasts (35) is dependent on tetrahydrobiopterin synthesis. Our studies confirm that cytokine-inducible NO synthase activity in rat aortic VSMC is dependent on tetrahydrobiopterin synthase. Treatment of RAVSMC with DAHP, an inhibitor of GTP cyclohydrolase, the rate-limiting enzyme in tetrahydrobiopterin biosynthesis, blocked the effect of IL-1, whereas addition of extracellular tetrahydrobiopterin, restored IL-1-induced cGMP in DAHP-treated VSMC, confirming that the action of DAHP was due to depletion of tetrahydrobiopterin. In contrast, DAHP and extracellular tetrahydrobiopterin had no effect on IL-1-induced cGMP in saphenous VSMC, indicating that the IL-1-inducible guanylate cyclase activating pathway in HSVSMC is independent of tetrahydrobiopterin.

Cytokine-activated macrophages, endothelial cells, and VSMC from rodents produce substantial amounts of NO which is oxidized to nitrite and nitrate, and these end-products accumulate extracellularly to micromolar levels. Whereas nitrite was present at ~4 μM in 48-h supernatants from human VSMC, IL-1 did not affect nitrite production. Interestingly, VSMC cultured from human mesenteric microarterioles have been reported to release NO in the basal state and this release is inhibited by L-NMMA (36). It is not clear whether NO produced by human VSMC in our study or in the latter study (36) is a product of an inducible or constitutive NO synthase.

Detection of inducible NO synthase mRNA by PCR indicates that IL-1-treated HSVSMC express very low levels of NO synthase mRNA compared with IL-treated RAVSMC (at least three orders of magnitude lower based on the number of cycles required to obtain visible product by ethidium bromide staining, and by quantitation of incorporated radioactivity into the relevant PCR product). IL-1-treated HSVSMC even contained lower levels of inducible NO synthase mRNA than nontreated RAVSMC. The finding of low levels of NO synthase mRNA suggests that an inducible NO synthase gene may be present in HSVSMC, although it was not effectively induced by IL1 under the experimental conditions employed. It remains to be determined whether the observed NO synthase related PCR product in HSVSMC represents an inducible NO synthase having significance in HSVSMC in vivo.

Whereas our results rule out a role of NO synthase in IL-1-induced cGMP in HSVSMC, activation of cGMP was inhibited by methylene blue and LY83,583, two inhibitors of endothelium-dependent relaxation that inhibit soluble guanylate cyclase activation independent of NO production. The mechanisms by which methylene blue and LY83,583 inhibit guanylate cyclase are not completely understood. Methylene blue may inhibit guanylate cyclase activation by oxidation of the regulatory hemoprotein moiety of the enzyme (37). Recent in vivo evidence indicates that methylene blue and LY83583 inactivate soluble guanylate cyclase by generating.

Figure 12. Effect of l-arginine analogs on cytokine-inducible cGMP in human umbilical artery (A) and rat vena cava (B) VSMC. (A) Human umbilical artery VSMC were incubated 6 h with or without IL1 (200 U/ml) and TNF (50 ng/ml) in MEM containing 50 μM l-arginine. IL-NMMA (1 mM) was added 40 min before and IBMX (1 mM) 20 min before cGMP determination. (B) Rat vena cava VSMC were incubated 24 h with or without IL1 (200 U/ml) in DMEM-5. 80 min before cGMP determination, VSMC were washed and the media replaced with MEM containing 50 μM l-arginine with or without l-arginine analogs (each 1 mM). IBMX (0.1 mM) was added 20 min before cGMP determination.
hydroxyl radical (38). Our results indicate that methylene blue and LY83,583 inhibit NO-independent as well as NO-dependent activation of soluble guanylate cyclase. The results also indicate that IL-1-induced cGMP in human VSMC involves NO-independent activation of soluble rather than particulate guanylate cyclase. IL-1-induced cGMP in human VSMC was not inhibited by hemoglobin which inhibits guanylate cyclase activation by endothelium-dependent vasodilators by binding NO with high affinity (39). These results may indicate that IL-1-treated human VSMC do not produce an activator of soluble guanylate cyclase which is bound by hemoglobin. However, we cannot rule out the possibility that hemoglobin is ineffective because it remains extracellular, and thus does not block the effect of a mediator which acts exclusively within the cell and not on adjacent cells.

Clear evidence has been presented for the regulation of vascular tone by constitutive endothelial NO synthase in humans. L-NMMA blocks acetylcholine-induced increases in forearm blood flow (5), and human endothelial NO synthase has been recently cloned from human umbilical vein endothelial cells (40). However, evidence concerning the existence and nature of a cytokine-inducible NO synthase in human vascular cells is limited. One report demonstrates IL-1-induced nitrite production in VSMC grown from a single human aorta (41), and inducible NO synthase transcripts have been reported in human aortic VSMC treated with a mixture of cytokines (16). Although in vivo studies also support the hypothesis that NO contributes to cytokine and sepsis-induced hypotension in humans, they are not definitive. t-arginine analogs were effective at improving blood pressure in early clinical trials with septic shock patients (42), however, blood pressure may improve in part because of inhibition of constitutive NO synthase. There is also evidence that cytokines induce NO synthase activity in humans: arginine-derived nitrate is increased in the plasma and urine of cancer patients receiving high-dose IL-2 therapy (43, 44), and is increased in the plasma of septic patients (45). However, the cellular source of the nitrate is not discernible from in vivo studies. Cytokine-inducible NO synthase has been demonstrated in only a few human cell types in vitro, in contrast to the abundance of nonhuman cell types shown to have cytokine-inducible NO synthase activity. Human hepatocytes (16) and human chondrocytes (46) express NO synthase activity after treatment with cytokines or LPS. However, cytokine treatment does not affect nitrite or nitrate accumulation in 24-48-h supernatants of human monocytes or macrophages (47-51). In one study, TNF stimulated nitrite accumulation in 7-d supernatants of human blood mononuclear cells (52), suggesting that nitrite may be produced but at very low levels. Our studies may indicate that cytokine-inducible NO synthase activity is not ubiquitous to all human VSMC. Alternatively, the experimental conditions which we used may not mimic those required for effective induction of the NO synthase gene in human cells. Regardless, our studies establish that IL-1 can induce a NO-independent pathway of guanylate cyclase activation in human VSMC, and thereby raise the question of the relative importance of NO-dependent and NO-independent pathways of guanylate cyclase activation in the vascular actions of cytokines in humans.

In conclusion, IL-1 increases cGMP in VSMC from human saphenous vein by a mechanism that is distinct from NO. In contrast to the IL-1-inducible NO pathway in RAVSMC, IL-1-inducible cGMP production in HSVSMC is not inhibited by NO synthase inhibitors including nitro- and methyl-substituted t-arginine analogs, aminoguanidine, and flavoprotein inhibitors, and is independent of extracellular t-arginine and tetrahydrobiopterin. IL-1 fails to enhance nitrite production by HSVSMC because of its inability to induce significant levels of NO synthase mRNA. This NO-independent pathway of guanylate cyclase activation appears to be specific to human VSMC, as it is also present in human umbilical artery VSMC, whereas VSMC from rat vena cava and rat aorta display NO-dependent increases in cGMP. It will be important to establish whether this distinct guanylate cyclase activating pathway is present in VSMC from other human arteries or veins, or in other human cell types. NO-independent activation of soluble guanylate cyclase in VSMC may contribute to systemic vasodilation in septic patients.

The authors gratefully acknowledge Dr. Jeffrey B. Tatro (Department of Medicine, New England Medical Center) for helpful comments and suggestions.

This work was supported by grants from Baxter Healthcare Corporation, Renal Division, Extramural Grant Program and the National Institutes of Health (HL-47569).

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Received for publication 14 April 1993 and in revised form 21 September 1993.

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