NO from smooth muscle cells decreases NOS expression in endothelial cells: role of TNF-α

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De Frutos, Trinidad, Lourdes Sánchez de Miguel, Margarita García-Durán, Fernando González-Fernández, Juan A. Rodríguez-Feo, Mercedes Montón, José Guerra, Jerónimo Farré, Santos Casado, and Antonio López-Farré. NO from smooth muscle cells decreases NOS expression in endothelial cells: role of TNF-α. *Am. J. Physiol.* 277 (*Heart Circ. Physiol.* 46): H1317–H1325, 1999.—Despite the evidence that cytokines stimulate nitric oxide (NO) production by inducible nitric oxide synthase (iNOS), several reports recently demonstrated that the hypotensive response related to endothelial nitric oxide synthase (eNOS) activity could be inhibited by the same cytokines. The aim of the present work was to analyze whether NO generated by vascular smooth muscle cells (VSMC) could modify eNOS protein expression in endothelial cells. Bovine aortic endothelial cells (BAEC) and bovine VSMC (BVSMC) in coculture were used for the study. Interleukin-1β (IL-1β, 10 ng/ml)-treated BVSMC, which expressed iNOS protein, decreased eNOS protein expression in BAEC. The presence of NO antagonists N^{ω}-nitro-L-arginine methyl ester (10^{-3} mol/l) or N^{G}-monomethyl-L-arginine (10^{-3} mol/l) prevented the decrease in eNOS protein expression induced by IL-1β-treated BVSMC. Surprisingly, two different NO donors, 3-morpholinosydnonimine (10^{-4} mol/l) and *S*-nitroso-*N*-acetyl-D,L-penicillamine (10^{-4} mol/l), failed to modify eNOS expression in BAEC, suggesting the existence of a diffusible mediator released from IL-1β-treated BVSMC that acts on endothelial cells by reducing eNOS expression. The presence of NO antagonists reduced tumor necrosis factor-α (TNF-α) production by IL-1β-stimulated BVSMC. This effect was also produced in the presence of a protein kinase G inhibitor, guanosine-5′-O-(2-thiodiphosphate) trilithium salt. A polyclonal antibody against TNF-α prevented eNOS expression in the BAEC-BVSMC coculture. In conclusion, NO by itself failed to modify eNOS protein expression in endothelial cells but increased TNF-α generation by IL-1β-stimulated BVSMC and, in this way, reduced eNOS expression in the endothelium.

coculture; endothelial dysfunction; interleukin-1β; nitric oxide donors; tumor necrosis factor-α

NITRIC OXIDE (NO) is a multifunctional molecule with an important role in the relations among the cells that make up the microvascular environment (21, 27). In the endothelium, the activity of an endothelial nitric oxide synthase (eNOS) mediates NO production. eNOS activity generates small amounts of NO for short periods of time and is dependent on calcium-increasing agents (20, 21). NO released by the eNOS activity is mainly responsible for the so-called endothelium-dependent vasorelaxing response (5).

eNOS activity could be regulated by a variety of factors, including cofactor availability and intracellular localization (10, 38). However, during the last few years different studies have suggested that the ability of the endothelium to produce NO could be also regulated by the level of eNOS expression (14, 18, 40).

It has been shown that vascular smooth muscle cells (VSMC) not only respond to NO but also are capable of producing it. Stimulation of VSMC with cytokines induces NO release from these cells by stimulating the expression of an inducible nitric oxide synthase (iNOS) isoform (4, 31). iNOS expression requires several hours, and its activity generates large amounts of NO over long periods of time (20, 21, 31). The induction of iNOS protein has been proposed to occur in a variety of inflammation-related cardiovascular diseases, e.g., cardiac allograft rejection, septic shock, heart failure, and atherosclerosis (9, 15, 34, 35), in which different cytokines, i.e., interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α), have been reported to be present (23, 30, 33).

In contrast to the evidence that cytokines stimulate NO production by iNOS activity, several reports recently demonstrated that the hypotensive response related to eNOS activity could be inhibited by the same cytokines (24, 25). In this regard, it was recently demonstrated that TNF-α reduces eNOS protein expression in cultured endothelial cells (1, 40). Moreover, the vasodilatation related to eNOS activity was shown to be impaired in atherosclerotic arteries, whereas the release of NO and cytokines was markedly increased (7, 19, 23, 35). Because NO by itself has been shown to regulate the expression and synthesis of several proteins, including growth factors, leukocyte adhesive proteins, and extracellular matrix proteins (11, 12, 41), the aim of the present work was to analyze whether NO generated by VSMC could modify eNOS protein expression in endothelial cells.

MATERIALS AND METHODS

Chemicals. Interleukin-1β, N^{ω}-nitro-L-arginine methyl ester (L-NAME) and N^{G}-monomethyl-L-arginine (L-NMMA) were purchased from Sigma (St. Louis, MO). 3-Morpholinosydnonimine hydrochloride (SIN-1) and *S*-nitroso-*N*-acetyl-D,L-penicillamine (SNAP) were obtained from Alexis (Läufelfingen, Switzerland). All other chemicals were of the highest commercially available quality from Sigma. The monoclonal

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antibodies against iNOS and eNOS were purchased from Transduction Laboratories (Lexington, KY). The polyclonal antibodies against TNF-α (10 ml neutralizes 1,000 U) and IL-1β (1 mg neutralizes 1,000 U) were purchased from Genzyme Diagnostics (Cambridge, MA).

Bovine aortic endothelial cell cultures. Bovine aortic endothelial cells (BAEC) were obtained and cultured as previously described (17). In brief, the aortic lumen was filled with 500 mg/l type II collagenase (Sigma) and incubated at 37°C for 20 min. BAEC were harvested in RPMI 1640 medium supplemented with 10% FCS, 5 mmol/l glutamine, 2×10^{-5} U/l penicillin, and 2×10^{-5} μg/l streptomycin. Cells were seeded into six-well plates and used at one or three passages. BAEC exhibited the typical cobblestone appearance and were positive for von Willebrand factor immunofluorescence.

Bovine VSMC cultures. After collagenase digestion to isolate the endothelium, the aortic lumen was scraped to remove contaminating endothelial material. Bovine aortic media tissue was then removed in small strips and transferred to tissue culture flasks containing the RPMI medium described in *Bovine aortic endothelial cell cultures*. Once bovine VSMC (BVSMC) migrated and grew from the explants, the cells were transferred to collagen type I-coated microporous membrane transwell inserts (0.4-μm pore size, Millipore). BVSMC were used at one and three passages. As described (16), the cells exhibited typical "hill and valley" growth morphology and reacted with anti-α-actin monoclonal antibody (Boehringer Mannheim).

BAEC-BVSMC coincubation experiments. The coculture system was prepared as previously described (16) by placing the transwell inserts containing the BVSMC into wells containing the BAEC. Before coincubation, BVSMC were stimulated with IL-1β (10 ng/ml, which corresponds to 0.03 U/l) for 8 h. BVSMC were then rinsed twice and cocultured with BAEC for a further period of 18 h. Therefore, only BVSMC were pretreated with IL-1β. To assess that IL-1β was efficiently washed from BVSMC, the level of IL-1β was determined in the last recovered washed medium by an ELISA kit (Boehringer Ingelheim, Vienna, Austria). The intra- and interassay variability of the IL-1β ELISA kit were 9.1 and 8.1%, respectively, and the low detection limit was 1.6 pg/ml. In the last recovered medium from the washed BVSMC, IL-1β was undetectable.

In this coculture system, the medium was shared by both types of cells. Humoral exchange was allowed between them without direct cell contact. This coculture technique permitted the further processing of BAEC alone. It should be noted that after BAEC and BVSMC were cocultured, the recovered medium remained free from microorganisms and endotoxin.

To analyze the effect of the NO released from BVSMC on eNOS expression in BAEC, the NO antagonists L-NAME (10^{-3} mol/l) or L-NMMA (10^{-3} mol/l) were added to the coculture system. In addition, the involvement of TNF-α was tested with an anti-TNF-α polyclonal antibody. As reported previously (32), the dilution of anti-TNF-α antibody (1:400) used was sufficient to completely neutralize TNF-α bioactivity in the standard L929 cell cytotoxicity.

Determination of iNOS and eNOS protein expression. eNOS protein expression was determined in BAEC coincubated with IL-1β-treated BVSMC as described in *BAEC-BVSMC coincubation experiments*. After the different experimental maneuvers, BAEC were collected and boiled in Laemmli buffer (13) containing 2-mercaptoethanol. Equal amounts of protein (20 μg/lane) measured by bicinchoninic acid reagent (Pierce, Rockford, IL) were loaded. To verify the equal amounts of proteins loaded in the gel, a parallel gel was run and stained with Coomassie blue and the intensities of the protein bands were examined. Proteins were separated on denaturing SDS-10% polyacrylamide gels and blotted onto nitrocellulose (Immobilon-P, Millipore). Blots were blocked overnight at 4°C with 5% nonfat dry milk in TBS-T (20 mmol/l Tris·HCl, 137 mmol/l NaCl, 0.1% Tween 20). Western blot analysis was performed with a monoclonal antibody against eNOS protein. Blots were incubated with the first antibody (1:2,500) for 1 h at room temperature and, after extensive washing, with the second antibody (horseradish peroxidase-conjugated anti-mouse immunoglobulin antibody) at a dilution of 1:1,500 for another hour. Specific eNOS protein was detected by enhanced chemiluminescence (ECL, Amersham) and evaluated by densitometry (Molecular Dynamics).

iNOS protein expression was determined in BVSMC following a similar protocol to the one described for eNOS detection but using a monoclonal antibody against iNOS protein. Prestained protein markers (Sigma) were used for molecular mass determinations. As previously reported (6, 18, 22), the monoclonal antibodies used specifically recognized the iNOS (130 kDa) and eNOS (140 kDa) isoforms and did not cross-react with the opposite NOS isoforms.

Measurement of NO production. NO release by the NO donors was assessed as nitrite generation. BAEC were incubated with the NO donors SIN-1 (10^{-4} mol/l) and SNAP (10^{-4} mol/l). After 18 h of incubation the supernatants were recovered, and after centrifugation (2,500 rpm, 10 min), nitrite accumulation was measured with a commercial kit (Cayman Chemical, Ann Arbor, MI) based in the Griess reaction after the conversion of nitrate to nitrite with nitrate reductase. Nitrite concentrations were determined at an optical density of 554 nm by comparison with standard solutions of sodium nitrite prepared in the same incubation culture medium.

The incubated medium was not completely free from nitrate; therefore, an aliquot of medium underwent the same process as the medium obtained from the cultured cells. The nitrite value obtained with the medium alone was used as a blank, and it was subtracted from all the samples.

TNF-α determinations. BVSMC were stimulated with IL-1β (10 ng/ml) for 18 h. In similar experiments, the L-arginine antagonists L-NMMA (10^{-3} mol/l) and L-NAME (10^{-3} mol/l), the NO donors SIN-1 (10^{-4} mol/l) and SNAP (10^{-4} mol/l), the cGMP analog dibutyryl cGMP (10^{-4} mol/l), and the protein kinase G inhibitor guanosine 5′-*O*-(2-thiodiphosphate) (GDP-β-S, 10^{-4} mol/l) were also added. TNF-α was measured in the BVSMC incubation medium by an ELISA Kit (Chromogenix). The intra- and interassay variability of the ELISA kit were 1.1 and 4.7%, respectively.

Statistical methods Results are expressed as means ± SE. Unless otherwise stated, each value corresponds to a minimum of six experiments done in triplicate. Comparisons were done by ANOVA or paired and unpaired Student's *t*-test when appropriate. Bonferroni's correction for multiple comparisons was used to determine the level of significance of the *P* value.

RESULTS

Effects of IL-1β-stimulated BVSMC on eNOS protein expression in BAEC. To analyze whether the NO produced by BVSMC could modify eNOS protein expression in adjacent BAEC, IL-1β-treated BVSMC plated on six-transwell inserts were placed over a monolayer of BAEC for 18 h. In these assays, BVSMC were rinsed twice before coincubation (see MATERIALS AND METHODS); therefore, only BVSMC were activated by the exogenous cytokine.

Despite the fact that BVSMC were only stimulated with IL-1β for 8 h, these cells still expressed the iNOS

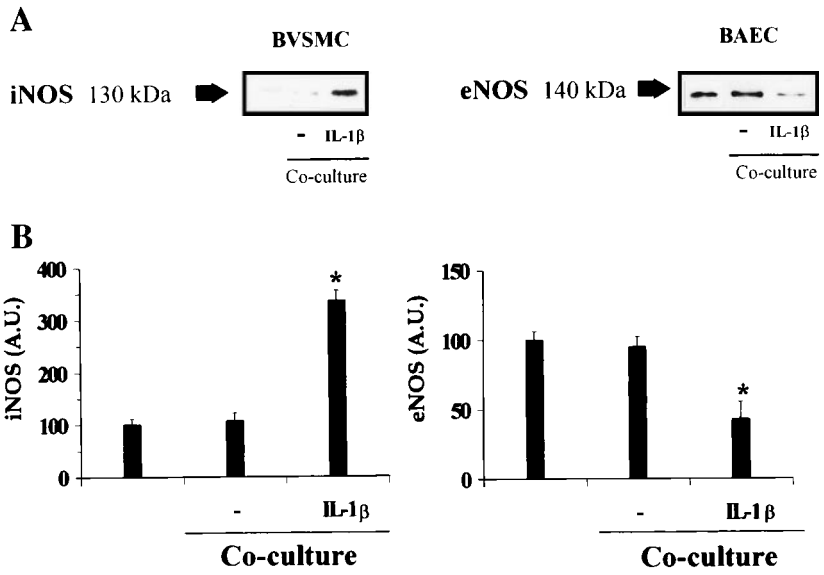

Fig. 1. *A*: representative Western blot that demonstrates expression of inducible nitric oxide synthase (iNOS) protein in bovine vascular smooth muscle cells (BVSMC, *left*) and endothelial nitric oxide synthase (eNOS) protein in bovine aortic endothelial cells (BAEC, *right*). BVSMC were preincubated for 8 h with or without interleukin-1β (IL-1β, 10 ng/ml), and, after being rinsed twice, they were coincubated with BAEC for a further 18 h. At this time, eNOS protein expression was determined in BAEC by Western blot. *B*: results of densitometric scan of 6 different Western blots in which basal value was determined as 100 arbitrary units (A.U.). Results are represented as means ± SE. $*P < 0.05$ with respect to basal conditions.

protein 18 h after being coincubated with BAEC (Fig. 1). The presence of IL-1β-treated BVSMC decreased eNOS protein expression in BAEC (Fig. 1), indicating that mediators released from IL-1β-treated BVSMC decreased eNOS protein expression in BAEC. Under basal conditions (BAEC coincubated with nontreated BVSMC), no effect of BVSMC on eNOS expression was observed different from that found in BAEC incubated alone (Fig. 1).

The next set of experiments was designed to demonstrate whether NO released by IL-1β-treated BVSMC could be involved in the reduction of eNOS expression observed in the adjacent BAEC. For this purpose, two different L-arginine competitors, L-NAME (10^{-3} mol/l) and L-NMMA (10^{-3} mol/l), were added to the coculture system. The above-mentioned decrease in eNOS protein expression in BAEC elicited by IL-1β-treated BVSMC was fully prevented by blocking NO generation with either L-NAME or L-NMMA (Fig. 2). In the absence of IL-1β- treated BVSMC, L-NAME and L-NMMA failed to modify eNOS protein expression in BAEC (Fig. 3).

Importance of BVSMC for effects of NO on eNOS protein expression in BAEC. Once we verified the decrease elicited by IL-1β-treated BVSMC on eNOS protein expression in BAEC and its prevention by the L-arginine antagonists, we further tested the ability of exogenous NO donors to reduce eNOS protein expression in BAEC.

Paradoxically, two different NO-generating compounds, SIN-1 (10^{-4} mol/l) and SNAP (10^{-4} mol/l), failed to reduce the expression of eNOS in BAEC, strongly suggesting that the presence of BVSMC is essential for the effects of NO on eNOS protein expression (Fig. 4, *A* and *B*). The ability of the NO donors to generate NO was assessed by measuring the nitrite concentration in the incubation medium. As shown in Fig. 4*C*, both SNAP and SIN-1 produced higher amounts of nitrite than those obtained in the coculture of BAEC with IL-β-treated BVSMC.

Therefore, the next hypothesis was that the NO released from IL-1β-treated BVSMC could act on the BVSMC themselves, releasing a diffusible factor that could be responsible for the decreased eNOS protein expression in BAEC.

TNF-α as mediator of interaction between BVSMC and BAEC. TNF-α was proposed as the mediator involved in the above-mentioned effects, on the basis of recent data that demonstrated that exogenous TNF-α reduced eNOS mRNA stability in endothelial cells (1, 40).

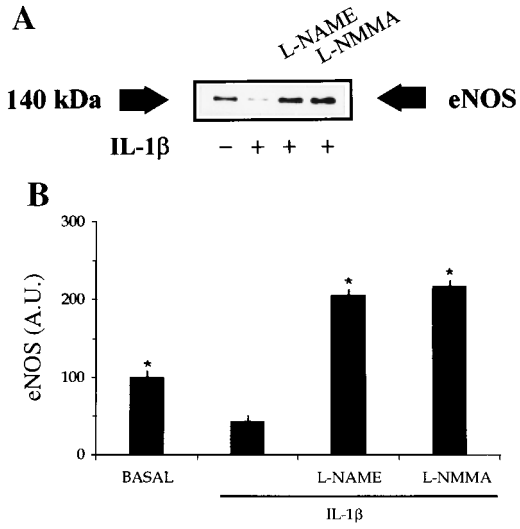

Fig. 2. *A*: representative Western blot demonstrating expression of eNOS protein in BAEC coincubated with IL-1β-treated BVSMC. BVSMC were preincubated for 8 h with IL-1β (10 ng/ml), and, after being rinsed twice, they were coincubated with BAEC for a further 18 h. In some experiments the L-arginine antagonists N^{ω}-nitro-L-arginine methyl ester (L-NAME, 10^{-3} mol/l) and N^{G}-monomethyl-L-arginine (L-NMMA, 10^{-3} mol/l) were also added. *B*: results of densitometric scan of 4 separate experiments in which basal value was determined as 100 A.U. Results are represented as means ± SE. $*P < 0.05$ with respect to BAEC incubated with IL-1β-treated BVSMC.

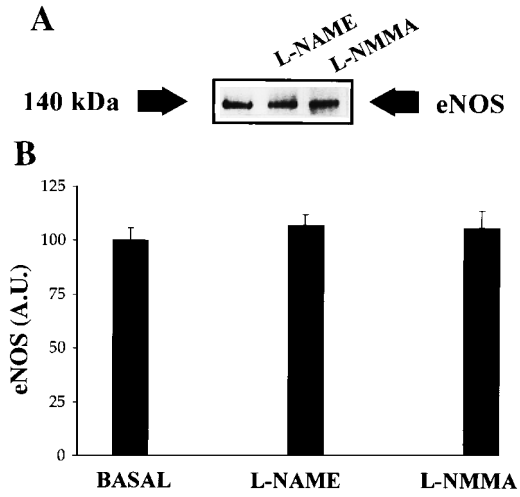

Fig. 3. *A*: representative Western blot demonstrating expression of eNOS protein in BAEC coincubated with BVSMC. L-arginine antagonists L-NAME (10^{-3} mol/l) and L-NMMA (10^{-3} mol/l) were added. *B*: results of densitometric scan of 3 separate experiments in which basal value was determined as 100 A.U. Results are represented as mean ± SE of 3 different experiments.

We determined whether an anti-TNF-α antibody could prevent the decrease in eNOS expression mediated by IL-1β-treated BVSMC. The addition of a polyclonal anti-TNF-α antibody to the BAEC-BVSMC coculture abolished the decreased eNOS protein expression in BAEC mediated by IL-1β-treated BVSMC (Fig. 5). This effect was not observed when a nonspecific IgG was used (Fig. 5). The efficacy of the polyclonal antibody to inhibit TNF-α activity was confirmed by its ability to prevent the reduction of eNOS protein expression produced by the addition of exogenous TNF-α even at concentrations >5,000 pg/ml (data not shown).

We then analyzed the ability of BVSMC to produce TNF-α. Under basal conditions the production of TNF-α by BVSMC was 22 ± 3 pg/ml, and it increased to 40 ± 2 pg/ml after IL-1β incubation. Because the reactivity of bovine TNF-α in the TNF-α ELISA used may not reach 100%, the values obtained could not be quantitative; therefore, they were expressed as percent change from the basal value. The addition of L-NAME (10^{-3} mol/l) or L-NMMA (10^{-3} mol/l) inhibited the release of TNF-α induced by IL-1β (Fig. 6*A*). Addition of the NO donors SIN-1 (10^{-4} mol/l) or SNAP (10^{-4} mol/l) alone did not modify the generation of TNF-α by BVSMC (Fig. 6*B*). However, these NO donors enhanced IL-1β-stimulated TNF-α production by BVSMC (Fig. 6*C*).

The addition of a cGMP analog, dibutyryl cGMP (10^{-4} mol/l), to IL-1β-stimulated BVSMC significantly enhanced TNF-α production by BVSMC (% increase in TNF-α released with respect to basal level: 46 ± 3; n = 5, $P < 0.05$). In the absence of IL-1β, the analog of cGMP failed to modify TNF-α production by BVSMC (% increase 4 ± 1; n = 5, P = not significant). A protein kinase G inhibitor, GDP-β-S (10^{-4} mol/l), reduced the release of TNF-α by IL-1β- stimulation of BVSMC in a degree similar to that observed with L-NAME and L-NMMA (% increase in TNF-α released with respect to basal level: 13 ± 4; n = 5, $P < 0.05$ with respect to IL-1β-stimulated BVSMC).

Finally, we tested the ability of exogenous TNF-α to decrease eNOS protein expression in BAEC. TNF-α reduced eNOS protein expression in BAEC in a dose-dependent manner (Fig. 7). The threshold dose of exogenous TNF-α to decrease eNOS protein was 50 pg/ml (Fig. 7), which was higher than that endogenously produced by IL-1β-stimulated BVSMC (40 ± 2 pg/ml), suggesting the involvement of other agents released from BVSMC, which could favor the effect of TNF-α on BAEC. In this regard, TNF-α has been reported to induce the release of other cytokines, including IL-1β, from VSMC (37). Therefore, we further determined the effect of exogenous IL-1β on eNOS protein expression in BAEC. The external addition of IL-1β diminished eNOS protein expression in a dose-dependent manner (Fig. 8). The threshold dose of exogenous IL-1β to decrease eNOS protein was >100 ng/ml (Fig. 8), which was higher than that of TNF-α (Fig. 7). Moreover, the same dose of IL-1β provoked a

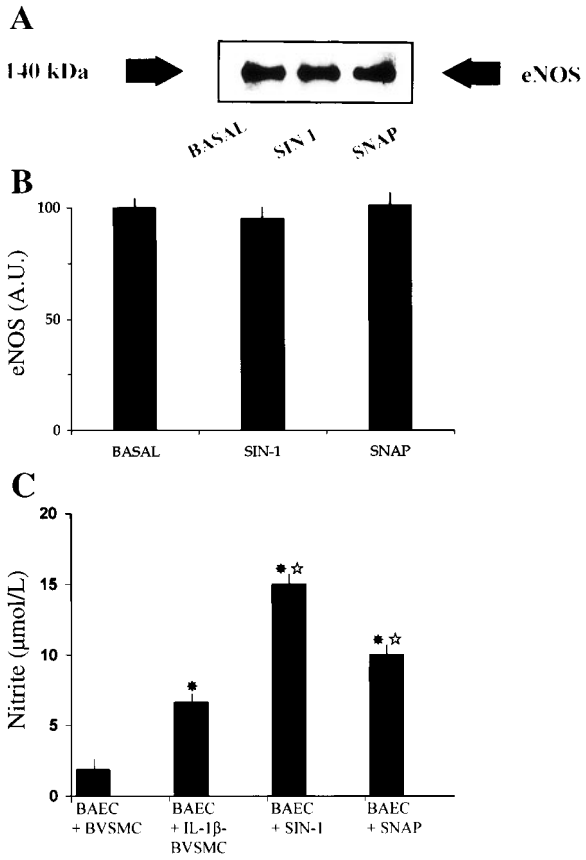

Fig. 4. *A*: representative Western blot that demonstrates expression of eNOS protein in BAEC incubated with 2 different NO donors, 3-morpholinosydnonimine (SIN-1, 10^{-4} mol/l) and *S*-nitroso-*N*-acetyl-D,L-penicillamine (SNAP, 10^{-4} mol/l). *B*: results of densitometric scan of Western blots of 5 different experiments in which basal value was determined as 100 A.U. *C*: nitrite production by BAEC incubated with BVSMC, IL-1β-treated BVSMC, or NO donor SIN-1 (10^{-4} M). Results are represented as means ± SE of 5 different experiments. *$P < 0.05$ with respect to BAEC-BVSMC. ☆$P < 0.05$ with respect to BAEC-IL-1β-treated BVSMC.

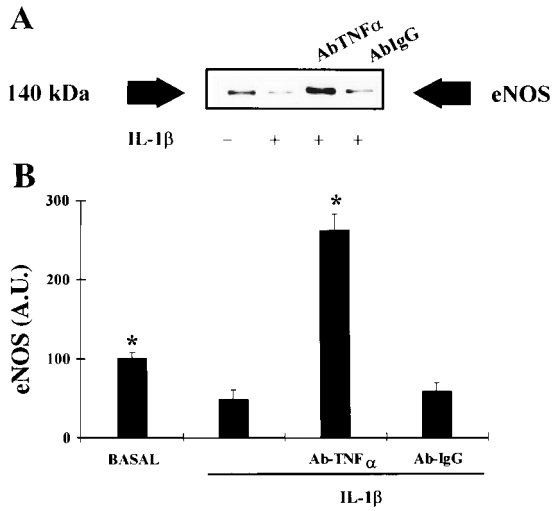

Fig. 5. *A*: representative Western blot that demonstrates effect of a polyclonal anti-TNF-α antibody (Ab) (1:400) on expression of eNOS protein in BAEC coincubated with IL-1β-treated BVSMC. BVSMC were preincubated for 8 h with IL-1β (10 ng/ml), and, after being rinsed twice, they were coincubated with BAEC for a further 18 h. During coculture, an anti-TNF-α Ab and nonspecific IgG were added. *B*: results of densitometric scan of 3 separate experiments in which basal value was determined as 100 A.U. Results are represented as means ± SE. $*P < 0.05$ with respect to BAEC coincubated with IL-1β-treated BVSMC.

significantly lesser reduction of eNOS protein expression than TNF-α in BAEC.

On the other hand, the addition of a polyclonal anti-IL-1β antibody (0.1 mg/ml) to the BAEC-BVSMC coculture inhibited the decrease in eNOS protein expression mediated by IL-1β-treated BVSMC (Fig. 9). The efficacy of the polyclonal antibody to inhibit IL-1β activity was confirmed by its ability to prevent the induction of iNOS protein expression by IL-1β-stimulated BVSMC (Fig. 10). It was noteworthy that the levels of eNOS expression achieved in the presence of anti-IL-1β were lower than in the presence of anti-TNF-α (Figs. 5*A* and 9*A*). In this regard, levels of anti-IL-1β antibody >0.1 mg/ml failed to increase its above-described effects on eNOS expression in the coculture of BAEC and BVSMC (data not shown).

DISCUSSION

The present study provides new evidence about the interaction of the two major constituents of the vascular wall, i.e., endothelial cells and smooth muscle cells. The main findings of the present work indicate that IL-1β-treated BVSMC decrease eNOS protein expression in BAEC by a mechanism in which NO and TNF-α are involved.

Several reports have shown that the vasodilator response to endothelial receptor-dependent agonists, which is related to eNOS activity, is substantially decreased after endotoxemia (23, 24), a pathological situation accompanied by increased iNOS activity in a variety of cell types, including VSMC (3, 31). In the same line of evidences, Minor et al. (19) showed an impaired endothelium-dependent vasorelaxation in atherosclerotic rabbits that was accompanied by an increased NO release from the vessel wall. Furthermore, Aoki et al. (2), by in vitro exposure of carotid arteries to TNF-α, a cytokine that stimulates iNOS gene expression (8), provided functional evidences of impaired agonist-dependent NO release. Moreover, NO by itself regulates the expression of several proteins (11, 12, 41). Therefore, we determined whether the NO released from VSMC through iNOS activity could affect eNOS protein expression in the adjacent endothelial cells. Our results showed that when iNOS protein expression was stimulated in BVSMC by IL-1β, eNOS protein expression in the coincubated BAEC was markedly reduced. The finding that eNOS protein expression was

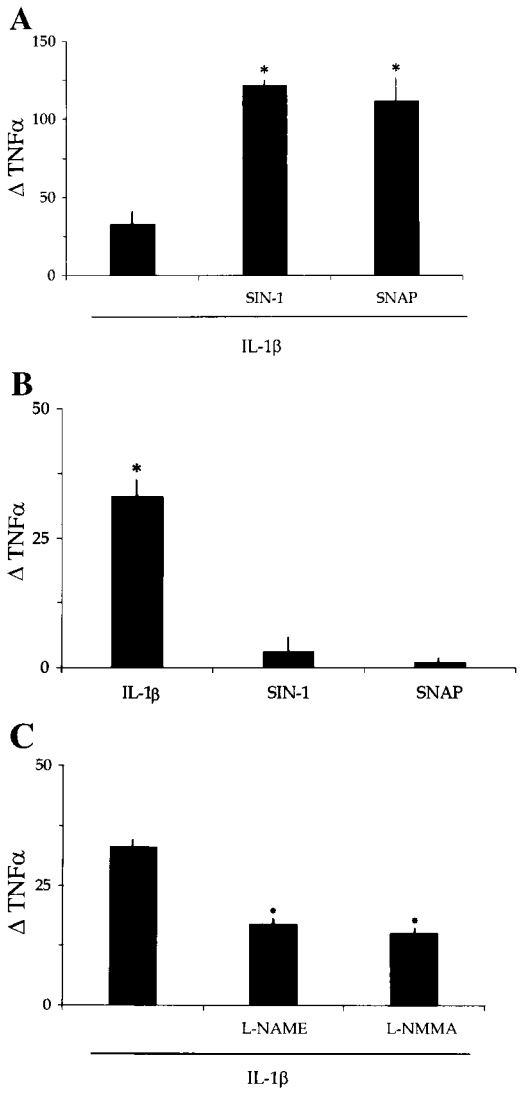

Fig. 6. *A*: TNF-α produced by IL-1β (0.03 U/l)-stimulated BVSMC in presence or absence of NO antagonists L-NAME (10^{-3} mol/l) and L-NMMA (10^{-3} mol/l). *B*: TNF-α produced by BVSMC incubated with IL-1β (0.03 U/l) or with 2 different NO donors, SIN-1 (10^{-4} mol/l) or SNAP (10^{-4} mol/l), for 18 h. *C*: TNF-α produced by IL-1β (10 ng/ml)-stimulated BVSMC in presence or absence of NO donors SIN-1 (10^{-4} mol/l) or SNAP (10^{-4} mol/l). Results are shown as increased TNF-α production (ΔTNF-α) with respect to basal production (22 ± 3 pg/ml) and are means ± SE of 6 different experiments. *A* and *C*: $*P < 0.05$ with respect to IL-1β-stimulated BVSMC. *B*: $*P < 0.05$ with respect to basal production.

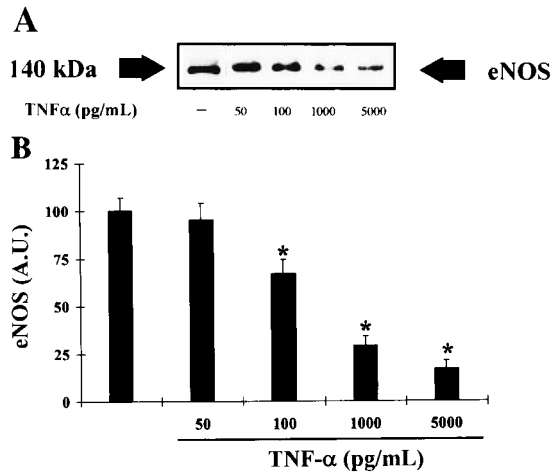

Fig. 7. *A*: representative Western blot demonstrating expression of eNOS protein in BAEC incubated in presence of increasing concentrations of TNF-α for 18 h. *B*: results of densitometric scan of 4 different Western blots in which basal value was determined as 100 A.U. Results are represented as means ± SE. $*P < 0.05$ with respect to basal conditions.

protected by antagonists of the L-arginine-NO pathway suggested that IL-1β-treated BVSMC decreased eNOS protein expression by NO-dependent mechanisms. It should be noted that the blockade of NO synthesis by IL-1β-treated BVSMC not only preserved but also increased eNOS protein expression in the endothelial cells above baseline levels.

Despite the fact that the incubation medium of the cells contained antibiotics and we failed to find bacteria and endotoxin in it, a weak iNOS protein expression in the absence of IL-1β was observed. This slight iNOS expression could be caused by agents contained in the FCS, i.e., cytokines.

Because both L-arginine antagonists, L-NAME and L-NMMA, reversed the effect of IL-1β-treated BVSMC on eNOS protein expression in endothelial cells, we

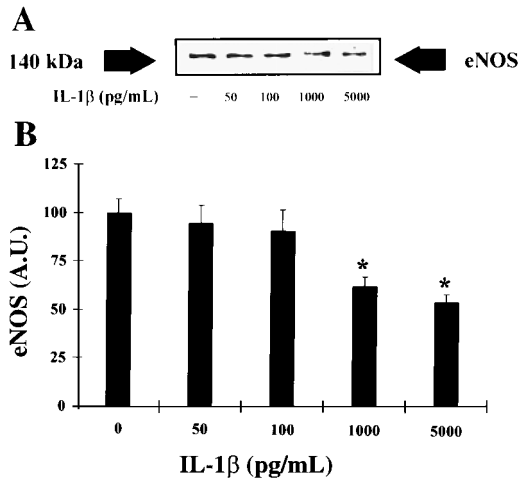

Fig. 8. *A*: representative Western blot demonstrating expression of eNOS protein in BAEC incubated in presence of increasing concentrations of IL-1β for 18 h. *B*: results of densitometric scan of 4 different Western blots, in which basal value was determined as 100 A.U. Results are represented as means ± SE. $*P < 0.05$ with respect to basal conditions.

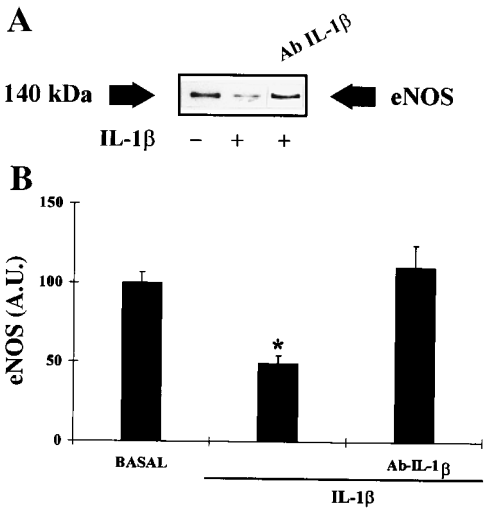

Fig. 9. *A*: representative Western blot demonstrating effect of a polyclonal anti-IL-1β Ab (0.1 mg/ml) on expression of eNOS protein in BAEC coincubated with IL-1β-treated BVSMC. BVSMC were preincubated for 8 h with IL-1β (10 ng/ml), and, after being washed twice, they were coincubated with BAEC for a further 18 h. During coculture, an anti-IL-1β Ab was added. *B*: results of densitometric scan of 3 separate experiments in which basal value was determined as 100 A.U. Results are represented as means ± SE. $*P < 0.05$ with respect to BAEC coincubated with IL-1β-treated BVSMC.

hypothesized that NO released from BVSMC could be the responsible mediator of the effects of BVSMC on eNOS decrease in the adjacent BAEC. To further support this suggestion, two different NO donors, SIN-1 and SNAP, were directly added to BAEC and eNOS protein expression was determined. Despite the fact that the amount of NO generated by the two NO donors was higher than the amount produced by the IL-1β-stimulated BVSMC, surprisingly, none of the NO donors modified eNOS protein expression in BAEC. Furthermore, in the absence of BVSMC, the blockade of

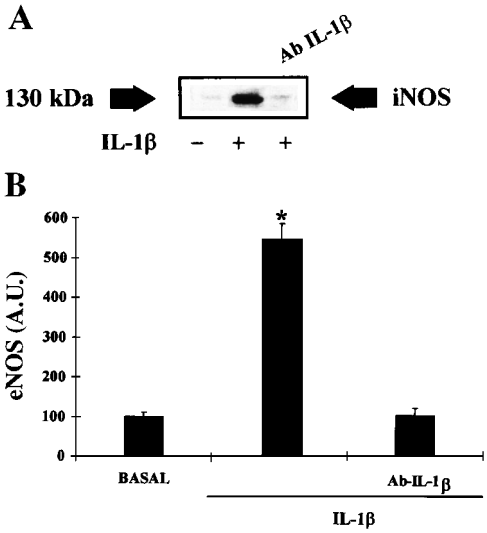

Fig. 10. Representative Western blot demonstrating effect of a polyclonal anti-IL-1β Ab (0.1 mg/ml) on expression of iNOS protein in IL-1β (10 ng/ml)-stimulated BVSMC. *B*: results of densitometric scan of 3 separate experiments in which basal value was determined as 100 A.U. Results are represented as means ± SE. $*P < 0.05$ with respect to basal conditions.

NO synthesis by BAEC failed to modify eNOS protein expression. These results suggested that the direct action of NO on endothelial cells was not the mechanism by which IL-1β-treated BVSMC decreased eNOS expression in the endothelial cells. These paradoxical results could indicate that NO released by BVSMC interacts with BVSMC themselves, favoring the production of other mediator(s) that act on endothelial cells, reducing the expression of the eNOS protein.

In the blood vessel, smooth muscle cells are a local source of TNF-α, which contributes substantially to the inflammatory response within the microenvironment of the vascular wall (28, 37). It was also demonstrated that TNF-α decreases eNOS expression in endothelial cells (40). In this regard, we recently demonstrated (1) that the reduction of eNOS mRNA stability, through the binding of endothelial cytosolic proteins to the 3′-unstranslated region of the eNOS mRNA, is the mechanism by which TNF-α regulates eNOS protein expression in BAEC. Therefore, TNF-α was proposed as a potential mediator involved in the observed BVSMC- and NO-dependent reduction of eNOS expression in endothelial cells. The addition of an anti-TNF-α antibody to BAEC-BVSMC cocultures completely prevented the inhibition of IL-1β-treated BVSMC on eNOS protein expression in endothelial cells.

Therefore, we determined whether NO produced by the cytokine-stimulated BVSMC modulates TNF-α production by these cells. We first observed that TNF-α production by BVSMC was stimulated in the presence of IL-1β. Furthermore, the two NO antagonists, L-NAME and L-NMMA, significantly decreased IL-1β-induced TNF-α production by BVSMC. However, NO was only a potentiator of TNF-α generation by IL-1β-stimulated BVSMC because it was unable by itself to stimulate TNF-α production in the absence of IL-1β. In this regard, the NO donors SIN-1 and SNAP enhanced IL-1β-induced TNF-α release by BVSMC.

The underlying mechanisms by which NO enhanced IL-1β-induced TNF-α release by BVSMC were further analyzed, keeping in mind that a main pathway of intracellular signal transduction of NO is cGMP (20, 21, 27). Moreover, it has been demonstrated that cGMP favors TNF-α production in macrophages by increasing TNF-α gene transcription (29). In the present work, the involvement of cGMP in the regulation of TNF-α production by BVSMC was suggested by the fact that a cGMP analog potentiated the release of TNF-α. Furthermore, a protein kinase G inhibitor reduced the release of TNF-α by IL-1β-stimulated BVSMC to a degree similar to that observed with the L-arginine antagonists.

It should also be noted that although L-NAME and L-NMMA partially inhibited TNF-α production by IL-1β-stimulated BVSMC, this was enough to maintain eNOS protein expression in BAEC, supporting the important role of NO released from BVSMC in the control of eNOS expression in endothelial cells.

An interesting observation was that the amount of exogenous TNF-α needed to directly reduce eNOS protein expression in BAEC was higher than that endogenously generated by IL-1β-stimulated BVSMC. The reactivity of bovine TNF-α in the TNF-α ELISA kit used was not 100%, and therefore we could not assess the exact amount of TNF-α generated by IL-1β-stimulated BVSMC. However, we could not discard the possibility that TNF-α produced by BVSMC could itself act on BVSMC and induce them to produce other agents that would contribute to sensitizing the endothelium to the action of endogenous TNF-α. In this sense, a number of studies have demonstrated that TNF-α induces the synthesis and release of agents such as cytokines and free radicals from smooth muscle cells (36, 37, 39). In this regard, IL-1β also diminished eNOS protein expression in the endothelium, although with a lesser ability than TNF-α, suggesting that NO and TNF-α are required but probably are not the only BVSMC-released mediators that decrease eNOS protein expression in the endothelium. Therefore, the regulation by cytokine-stimulated smooth muscle cells of the eNOS expression in the endothelium could be a multifactorial phenomenon, in which TNF-α and NO could be some of the main mediators. Further studies are needed to clarify whether other inhibitors released from the smooth muscle cells might be implicated in the above-described effects.

In conclusion, we have shown that IL-1β-stimulated BVSMC reduce eNOS protein expression in endothelial cells by at least an NO- and TNF-α-dependent mechanism. The loss of eNOS expression by the endothelium and, therefore, the resulting decrease in its capability to produce NO in response to physiological stimuli, could compromise the ability of these cells to protect against thrombosis and leukocyte adhesion and favor the impaired endothelium-dependent hypotensive response described in a variety of cardiovascular diseases (20, 21, 26, 27). Increased levels of cytokines, which are stimulators of iNOS expression, have been also demonstrated in these pathological situations (23, 30, 33), in which an endothelial dysfunction seems to have a main role in their development. Thus the present work points out the potential importance of VSMC in the regulation of eNOS protein expression in endothelial cells and, therefore, the putative involvement of VSMC in the regulation of inflammation-related endothelial dysfunction.

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