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Regulation of Interleukin-1β-Stimulated Inducible Nitric Oxide Synthase Expression in Cultured Vascular Smooth Muscle Cells by Hemostatic Proteins

William Durante,†‡ Michael H. Kroll,**
Gregory J. Orloff,§ James M. Cunningham,§
*Timothy Scott-Burden,‖ Paul M. Vanhoutte‖ and Andrew I. Schafer**

*Medical Service, Houston VA Medical Center, and the Department of Medicine and †Pharmacology, Baylor College of Medicine, and ‖Center for Experimental Therapeutics, Baylor College of Medicine, Houston, TX 77030; and §Division of Hematology-Oncology, Department of Medicine, Brigham and Women's Hospital, Boston, MA 02115, U.S.A.

ABSTRACT. Experiments were performed to examine the mechanism by which specific hemostatic proteins regulate the release of nitric oxide (NO) from interleukin-1β (IL-1β) stimulated cultured rat aortic smooth muscle cells. Treatment of smooth muscle cells with IL-β stimulated inducible nitric oxide synthase (iNOS) mRNA expression, which preceded the release of NO (as measured by the accumulation of nitrite in the culture media). The cytokine-stimulated production of nitrite was blocked by the protein synthesis inhibitor cycloheximide, the transcriptional inhibitor actinomycin D, and the competitive inhibitor of NOS nitro-L-arginine. However, only actinomycin D inhibited IL-1β-stimulated iNOS mRNA expression. Treatment of smooth muscle cells with IL-1β in the presence of platelet derived growth factor or thrombin resulted in the inhibition of cytokine-stimulated expression of iNOS mRNA and NO release. The inhibitory effect of thrombin was reversed by hirudin and was mimicked by a 14 amino acid thrombin receptor activating peptide. In contrast, the concomitant exposure of smooth muscle cells to IL-1β and plasmin resulted in the potentiation of both IL-1β-stimulated iNOS expression and NO generation. Finally, treatment of smooth muscle cells with IL-1β in the presence of the hemostatic proteins did not affect the half-life of iNOS mRNA. These results demonstrate that specific protein components of the hemostatic system regulate IL-1β-stimulated iNOS mRNA expression in vascular smooth muscle cells. The capacity of hemostatic proteins to modulate the induction of vascular iNOS activity may play an important role in governing the release of NO and regulating thrombogenesis *in vivo*. BIOCHEM PHARMACOL 51;6:847–853, 1996.

KEY WORDS. smooth muscle; nitric oxide; hemostasis

NO[¶] is an important intercellular messenger molecule involved in a variety of physiological processes [1]. In the circulation, the release of NO plays a pivotal role in regulating blood flow by inhibiting smooth muscle contraction [2] and platelet aggregation [3, 4]. NO is synthesized from the terminal guanidino nitrogen atoms of L-arginine by the action of a family of enzymes designated NOS. At least two distinct classes of NOS have been identified. One class of the enzyme is constitutively active and is regulated by calcium and calmodulin, whereas the other class is expressed only after transcriptional initiation and its activity is calcium/calmodulin independent [5]. The latter enzyme is termed iNOS and is present in many cell types including macrophages, hepatocytes, cardiac myocytes, endothelial cells, and vascular smooth muscle cells [6–12]. Recently, iNOS has been purified and cloned from mouse macrophages [5, 13, 14] and from rat aortic smooth muscle cells [15, 16]. Sequence comparison of the isolated cDNAs show between 93 and 97% homology, suggesting that these are isoforms produced by genetic polymorphisms.

Exposure of vascular smooth muscle cells to IL-1β and other inflammatory mediators induces these cells to produce NO [10–12]. This cytokine-mediated release may have important pathophysiological and pharmacological consequences. The induced production of NO by smooth muscle cells inhibits blood vessel contractility and may contribute to the peripheral vascular collapse observed in Gram-negative sepsis [17, 18] and in the hypotension observed in patients undergoing antineoplastic therapy with IL-2 [19]. In addition, the release of NO by smooth muscle cells may play an important hemostatic role at sites of vascular injury since we have shown recently that IL-1β-treated smooth muscle cells inhibit platelet activation through the release of NO [20].

Induction of NOS in vascular smooth muscle cells involves

‡ Corresponding author: William Durante, Ph.D., Houston VA Medical Center, Building 109, Room 116, 2002 Holcombe Blvd., Houston, TX 77030. Tel. (713) 794-7086; FAX (713) 794-7618.

[¶] *Abbreviations:* NO, nitric oxide; (i)NOS, (inducible) nitric oxide synthase; IL-1β, Interleukin-1β; MEM, minimum essential medium; CU, Caseinolytic Units; PDGF, platelet derived growth factor; and CsCl, cesium chloride.

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de novo protein synthesis [10, 11, 20] and is impaired by anti-inflammatory corticosteroids [18]. The release of NO from IL-1β-activated smooth muscle cells is also inhibited by platelets via the release of PDGF [21]. Furthermore, thrombin and plasmin, the major proteases of the coagulation and fibrinolytic systems, respectively, exert divergent regulatory effects on the cytokine-mediated production of NO [22, 23]. These findings suggest a model in which components of the hemostatic system coordinately regulate the release of NO from vascular smooth muscle cells exposed to inflammatory mediators. The present study was designed to elucidate the molecular mechanism(s) by which IL-1β-induced NOS activity is regulated by hemostatic proteins.

MATERIALS AND METHODS

Materials

Fetal bovine serum, L-glutamine, cylcoheximide, trypsin, EDTA, hirudin, penicillin, streptomycin, sodium citrate, sodium sarcosinate, sodium nitrite, *N*-(1-naphthyl)ethylenediamine dihydrochloride, sulfanilamide, collagenase, elastase, trypan blue, and monoclonal antibodies against smooth muscle α-actin were purchased from the Sigma Chemical Co. (St. Louis, MO); guanidine isothiocyanate and CsCl were from GIBCO (Grand Island, NY); nitro-L-arginine was from the Aldrich Chemical Co. (Milwaukee, WI); human α-thrombin (3022 NIH U/mg) was from US Biochemicals (Cleveland, OH); thrombin receptor activating peptide, SFLLRNPNDKYEPF (T-14), was synthesized at the Baylor College of Medicine Protein Chemistry Core Facility; human plasmin (18.6 CU/mL) and S-2251 chromogenic substrate were from Kabi (Stockholm, Sweden); MEM was from ICN Biomedicals Inc. (Costa Mesa, CA); limulus amebocyte lysate and endotoxin standards were from the Associates of Cape Cod (Woods Hole, MA); PDGF-AB, actinomycin D, ribonuclease-A and ribonuclease-T1 were from Boehringer Mannheim (Indianapolis, IN); murine recombinant IL-1β (1×10^7 U/mg) was from R & D Systems (Minneapolis, MN); and [α-^{32}P]UTP (400 Ci/mmol) was from Amersham (Arlington Heights, IL).

Cell Culture

Smooth muscle cells were isolated by elastase and collagenase digestion of rat thoracic aorta and characterized by morphological criteria and by indirect immunofluorescent staining for smooth muscle α-actin using mouse anti-α-actin antibody immunoglobulin G fluorescein isothiocyanate conjugate [24]. Cells were cultured serially in MEM containing Earle's salts, 5.6 mM glucose, 2 mM L-glutamine, 20 mM Tris–NaOH, 20 mM HEPES–NaOH, 10% (v/v) heat-inactivated fetal bovine serum, 100 U/mL penicillin, and 100 U/mL streptomycin. Tissue culture media were screened for endotoxin contamination using a quantitative limulus amebocyte lysate assay and found to contain less than 0.16 ng/mL of endotoxin. Subcultured strains of smooth muscle cells were used in passages 10–33. Smooth muscle cells were seeded into either 12-well multiwell plates (Corning Inc., Corning, NY) or 100-mm culture dishes (Costar Corp., Cambridge, MA). When cells reached confluence (3 days after plating), the culture media were replaced with serum-free MEM containing bovine serum albumin (0.1% w/v) for 48 h to render the cells quiescent. Smooth muscle cells were then exposed to IL-1β or its vehicle in the presence or absence of specific compounds for various periods of time. Following incubation, supernatants were collected for nitrite measurement, and total RNA was extracted for ribonuclease protection analysis.

In some experiments, viability of smooth muscle cells was determined by trypan blue exclusion and found to be greater than 96%. Treatment of smooth muscle cells with the various reagents was restricted to those concentrations that did not adversely affect cell viability.

Production of NO from Smooth Muscle Cells

The presence of NO in smooth muscle cell culture supernatants was determined by measuring the amount of nitrite, a stable oxidation product of NO [25]. Aliquots (400 μL) of incubation media were collected and mixed with an equal volume of Griess reagent [1% sulfanilamide-0.1% *N*-(1-naphthyl)ethylenediamine dihydrochloride in 2% phosphoric acid] [26]. The mixture was incubated at room temperature for 10 min, and then the absorbance at 540 nm was measured (Ultraspec III, Pharmacia, Uppsala, Sweden). Concentrations were determined relative to a standard curve by using an aqueous solution of sodium nitrite, and background nitrite values corresponding to serum-free medium were subtracted from experimental values.

RNA Isolation and Ribonuclease Protection Analysis

Total cellular RNA was obtained by the guanidine isothiocyanate/CsCl gradient method [27]. Briefly, monolayers of smooth muscle cells were washed once in phosphate-buffered saline and lysed in guanidine isothiocyanate (4 M) containing sodium citrate (25 mM) and sodium sarcosinate (0.5%; w/v). The lysate was layered on a CsCl (5.7 M) cushion and ultracentrifuged at 100,000 *g* for 22 hr (Beckman Instruments Inc., model L8-80M, Palo Alto, CA). The RNA pellet was dissolved in Tris (10 mM)–EDTA (1 mM) buffer (pH 7.6) and then extracted with phenol:chloroform:isoamyl alcohol (25:24:1) followed by chloroform:isoamyl alcohol (24:1). Total RNA was precipitated with sodium acetate (3 M; pH 5.2) and ethanol, dried, and redissolved in Tris–EDTA buffer. RNA concentration was quantified by measuring absorbance at 260 nm.

For the ribonuclease protection assay, a rat aortic smooth muscle iNOS (160 base pair restriction fragment) [16] cRNA probe, labeled with [α-^{32}P]UTP by *in vitro* transcription using T3 RNA polymerase (Ambion Inc., Austin, TX), was used. A radiolabeled antisense RNA probe for mouse β-actin (249 base pair restriction fragment: Ambion Inc.) was also employed to control for variations in the amount of RNA used in each assay. Hybridization reactions were performed with 10 μg of RNA in 55% formamide for 16 hr at 48°C using 0.5 to 2.0 ×

10^5 cpm of radiolabeled cRNA per reaction, followed by digestion with ribonuclease-A (40 μg/mL) and -T1 (2 μg/mL) at room temperature for 30 min. Protected RNA was analyzed by electrophoresis using a 6% acrylamide/8 M urea gel. The gel was exposed overnight to X-ray film at −70°C in the presence of intensifying screens. The predicted base pair protected fragments of iNOS and β-actin mRNA were confirmed using a DNA sequence ladder as a molecular size marker. In some experiments, the density of the autoradiographic signals for iNOS was quantified by transmittance densitometry (LKB 2222-020 Ultrascan XL Laser Densitometer, Bromma, Sweden) and normalized with respect to the β-actin mRNA.

Plasmin Preparation

Plasmin was dissolved in distilled water and stored in aliquots of 100 CU/mL. Plasmin activity was determined using the S-2251 chromogenic substrate assay. The assay was performed at 37°C in an aggregometer (Bio Data model PAP-4C, Horsham, PA) equipped with a chromogenic program, as previously described [24].

Statistical Analysis

Results are expressed as means ± SEM. Statistical analysis was performed with the use of Student's two-tailed paired *t*-test and of an analysis of variance when more than two treatments were compared. P values less than 0.05 were considered to be statistically significant.

RESULTS

Treatment of rat aortic smooth muscle cells with IL-1β (2 ng/mL) stimulated the expression of iNOS mRNA. The mRNA was detectable 2 hr after stimulation, became maximal at 4 hr, and then gradually declined over time, nearly disappearing by 24 hr (Fig. 1: inset). In unstimulated smooth muscle cells, no mRNA signal was detectable (Fig. 1: inset). No significant changes in β-actin mRNA levels were observed during IL-1β treatment (Fig. 1: inset). IL-1β treatment also stimulated the production of nitrite from smooth muscle cells in a time-dependent manner. Nitrite formation followed the appearance of the iNOS transcript such that a significant increase in nitrite over unstimulated levels was first observed after 6 hr and an approximately 10-fold increase was seen after 24 hr of stimulation (Fig. 1).

Based on the time–course experiments which indicated that maximal levels of iNOS mRNA and nitrite were detected at 4 and 24 hr, respectively, these time intervals were used in all subsequent experiments. The increase of iNOS mRNA and nitrite release was dependent on the concentration of IL-1β (Fig. 2). The protein synthesis inhibitor cycloheximide (5 μg/mL) enhanced IL-1β-stimulated iNOS mRNA expression, whereas the transcriptional inhibitor actinomycin D (2 μg/mL) blocked iNOS mRNA induction (Fig. 3: inset). Both agents significantly attenuated the IL-1β-mediated nitrite release (Fig. 3). Nitro-L-arginine (300 μM) completely inhibited nitrite synthesis, but had no effect on IL-1β-induced mRNA levels (Fig. 3).

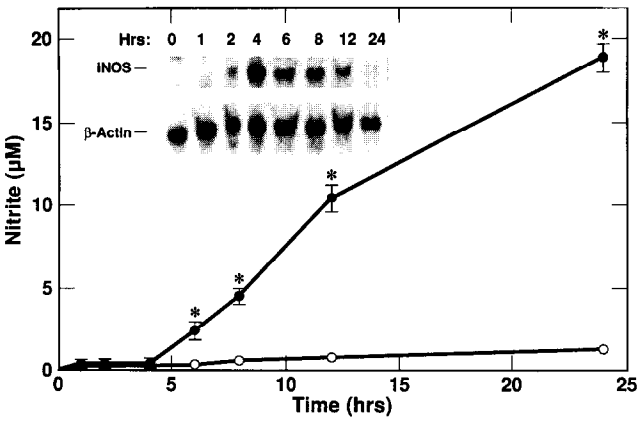

FIG. 1. Time course of smooth muscle cell iNOS expression following IL-1β exposure. Nitrite production was measured from unstimulated (○) and IL-1β (2 ng/mL) stimulated (●) cultured rat aortic smooth muscle cells. Results are the means ± SEM of three separate experiments performed in duplicate. Key: (*) statistically significant effect ($P < 0.05$) of IL-1β treatment. Inset: Ribonuclease protection analysis of iNOS and β-actin mRNA from cultured rat aortic smooth muscle cells stimulated with IL-1β (2 ng/mL; 0–24 hr). Similar findings were obtained in two separate experiments.

The simultaneous treatment of smooth muscle cells with IL-1β (3 ng/mL) and PDGF (3–30 ng/mL) resulted in a parallel inhibition of the cytokine-stimulated expression, both of iNOS mRNA and of nitrite release that was dependent on PDGF concentration (Fig. 4). In the absence of IL-1β, PDGF

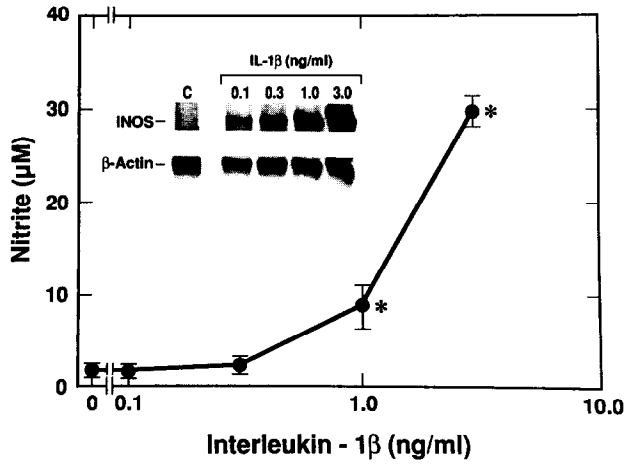

FIG. 2. Concentration–response curve of smooth muscle cell iNOS expression following IL-1β exposure. Nitrite production was measured from IL-1β (0.1 to 3.0 ng/mL for 24 hr) stimulated cultured rat aortic smooth muscle cells. Results are the means ± SEM of three separate experiments performed in duplicate. Key: (*) statistically significant effect ($P < 0.05$) of IL-1β treatment. Inset: Ribonuclease protection analysis of iNOS and β-actin mRNA from cultured rat aortic smooth muscle cells stimulated with IL-1β (0.1 to 3.0 ng/mL for 4 hr) and from unstimulated (C) cells. Similar findings were obtained in three separate experiments.

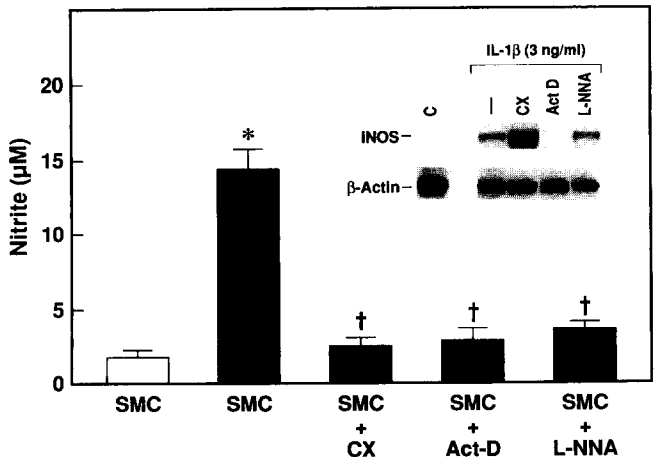

FIG. 3. Effect of cycloheximide (CX), actinomycin D (Act-D), and nitro-L-arginine (L-NNA) on smooth muscle cell iNOS expression following IL-1β exposure. Nitrite production was measured from unstimulated (open bar) and IL-1β (3.0 ng/mL for 24 hr) stimulated (closed bars) cultured rat aortic smooth muscle cells in the presence and absence of CX (5 μg/mL), Act-D (2 μg/mL), or L-NNA (300 μM). Results are the means ± SEM of three separate experiments performed in duplicate. Key: (*) statistically significant increase ($P < 0.05$) in nitrite release compared with untreated control cells; and (+) statistically significant inhibitory effect ($P < 0.05$) on IL-1β-stimulated release of nitrite. Inset: Ribonuclease protection analysis of iNOS and β-actin mRNA from unstimulated (C) and from stimulated (IL-1β; 3.0 ng/mL for 4 hr) cultured rat aortic smooth muscle cells in the presence and absence of CX (5 μg/mL), Act-D (2 μg/mL), or L-NNA (300 μM). Similar findings were observed in three separate experiments.

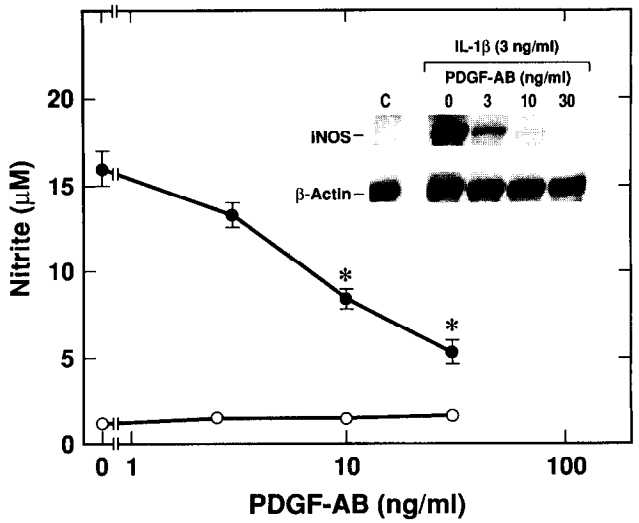

FIG. 4. Effect of PDGF on smooth muscle cell iNOS expression following IL-1β exposure. Nitrite production was measured from unstimulated (○) and IL-1β (3.0 ng/mL for 24 hr) stimulated (●) cultured rat aortic smooth muscle cells in the presence and absence of PDGF-AB (1–30 ng/mL). Results are the means ± SEM of three separate experiments performed in duplicate. Key: (*) statistically significant effect ($P < 0.05$) of PDGF treatment on IL-1β-stimulated nitrite release. Inset: Ribonuclease protection analysis of iNOS and β-actin mRNA from unstimulated (C) and from IL-1β (3.0 ng/mL for 4 hr) stimulated cultured rat aortic smooth muscle cells in the presence and absence of PDGF-AB (3–30 ng/mL). Similar findings were observed in three separate experiments.

had no effect on nitrite release (Fig. 4) or on iNOS mRNA expression (data not shown). Similarly, the simultaneous exposure of smooth muscle cells to IL-1β (3.0 ng/mL) and thrombin (0.1 to 3.0 U/mL) resulted in a thrombin concentration-dependent inhibition of both IL-1β-mediated iNOS mRNA expression and of nitrite synthesis (Fig. 5). The inhibitory effects of thrombin were reversed completely by treating thrombin with a specific thrombin inhibitor, hirudin (30 U/mL), and the addition of T-14 (100 μM) to smooth muscle cells inhibited the release of nitrite and the expression of iNOS message induced by IL-1β (Fig. 6), demonstrating that the thrombin effect was a specific, receptor-mediated response. In the absence of IL-1β, thrombin and T-14 had no effect on smooth muscle cell nitrite production or on iNOS mRNA expression (data not shown).

Plasmin alone minimally affected nitrite release from smooth muscle cells, and in all instances, except one, plasmin did not stimulate iNOS mRNA. In one experiment, prolonged autoradiographic exposure revealed a faint iNOS transcript that was induced by a high concentration of plasmin (1 CU/mL) (data not shown). The simultaneous exposure of smooth muscle cells to plasmin (0.1 to 1.0 CU/mL) and IL-1β (1.0 ng/mL) resulted in a plasmin concentration-dependent potentiation of both IL-1β-stimulated iNOS mRNA expression and of nitrite generation (Fig. 7).

In a final series of experiments, the decay in IL-1β-induced iNOS mRNA was monitored both in the presence and absence of PDGF (30 ng/mL), thrombin (3 U/mL), or plasmin (1 CU/mL) after adding actinomycin D (2 μg/mL) to arrest transcription. The half-life of iNOS mRNA stimulated with IL-1β alone was about 2–3 hr. Co-stimulation with the various hemostatic proteins did not change significantly the message half-life (Fig. 8).

DISCUSSION

The present study demonstrated that IL-1β induces the expression of the iNOS gene in vascular smooth muscle cells. This is shown by the cytokine-mediated stimulation of iNOS mRNA production and the subsequent generation of NO. IL-1β-induced vascular smooth muscle cell iNOS mRNA expression depends on cytokine concentration and duration of exposure. The induction of iNOS mRNA precedes the formation of NO, which correlates with NOS enzymatic activity as determined by nitrite levels in the culture media. Inducible NOS mRNA was not detected in unstimulated cells, but was observed within 2 hr of IL-1β treatment, suggesting that the regulation of smooth muscle cell iNOS activity in response to IL-1β is primarily transcriptional, as previously reported with macrophages iNOS [5, 13]. The inhibition of iNOS mRNA by actinomycin D observed in these studies further supports this notion.

The addition of the specific NOS inhibitor nitro-L-arginine

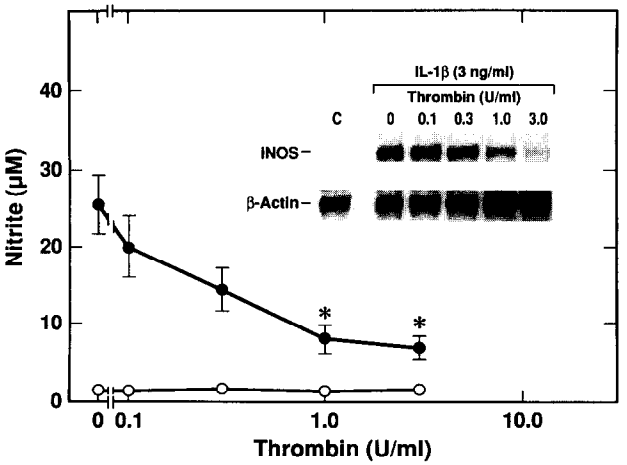

FIG. 5. Effect of thrombin on smooth muscle cell iNOS expression following IL-1β exposure. Nitrite production was measured from unstimulated (○) and IL-1β (3.0 ng/mL for 24 hr) stimulated (●) cultured rat aortic smooth muscle cells in the presence and absence of thrombin (0.1 to 3.0 U/mL). Results are the means ± SEM of three separate experiments performed in duplicate. Key: (*) statistically significant effect ($P < 0.05$) of thrombin treatment on IL-1β-stimulated nitrite release. Inset: Ribonuclease protection analysis of iNOS and β-actin mRNA from unstimulated (C) and from IL-1β (3.0 ng/mL for 4 hr) stimulated rat aortic smooth muscle cells in the presence and absence of thrombin (0.1 to 3.0 U/mL). Similar findings were observed in three separate experiments.

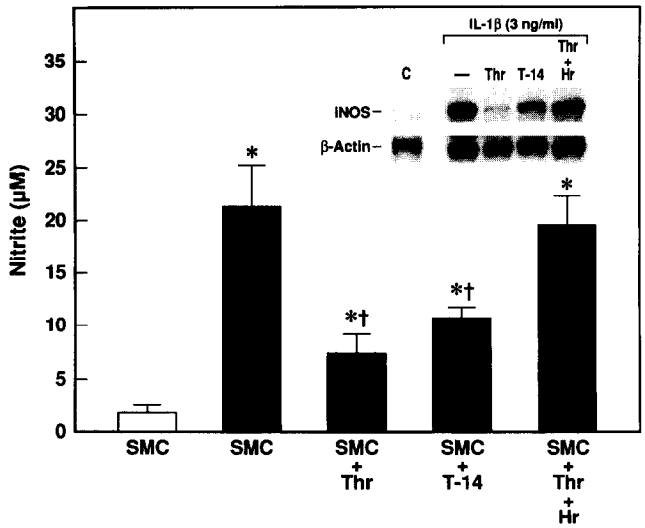

FIG. 6. Characterization of the inhibitory effect of thrombin (Thr) on smooth muscle cell iNOS expression following IL-1β exposure. Nitrite production was measured from unstimulated (open bar) and IL-1β (3.0 ng/mL for 24 hr) stimulated (closed bars) cultured rat aortic smooth muscle cells in the presence and absence of Thr (3.0 U/mL), thrombin receptor activating peptide (T-14; 100 μM), or the combination of Thr (3.0 U/mL) and hirudin (Hr; 30 U/mL). Results are the means ± SEM of four separate experiments performed in duplicate: Key: (*) statistically significant effect ($P < 0.05$) of IL-1β treatment; and (+) statistically significant inhibitory effect ($P < 0.05$) on IL-1β-stimulated release of nitrite. Inset: Ribonuclease protection analysis of iNOS and β-actin mRNA from unstimulated (C) and from stimulated (IL-1β; 3 ng/mL for 4 hr) cultured rat aortic smooth muscle cells in the presence and absence of Thr (3.0 U/mL), T-14 (100 μM), or the combination of Thr (3.0 U/mL) and Hr (30 U/mL). Similar findings were observed in three separate experiments.

[28] blocked the IL-1β-stimulated release of nitrite, indicating that NOS activity is responsible for the generation of nitrite, while having no effect on cytokine-induced mRNA levels. Treatment of smooth muscle cells with cycloheximide prevented IL-1β-stimulated release of nitrite, demonstrating that NO generation is dependent on *de novo* protein synthesis. In contrast to its effect on nitrite production, however, cycloheximide potentiated cytokine-induced iNOS mRNA levels. This probably reflects the well-described "superinduction" phenomenon that has been demonstrated for several growth-related primary response genes (i.e. c-*fos*, c-*myc*) and is thought to involve a transient increase in transcription accompanied by an increase in mRNA stability [29, 30]. Our observation of the "superinduction" phenomenon in vascular smooth muscle cells contrasts with that observed in macrophages and hepatocytes, where cycloheximide blocks iNOS mRNA induction [31, 32]. This suggests that iNOS mRNA expression is differentially regulated in different cell types.

Once expressed, iNOS generates large quantities of NO [1]. If this enzymatic activity were not regulated, the release of large amounts of NO into the circulation could have deleterious effects, such as excessive vasodilation and the inhibition of platelet thrombus formation at sites of vascular injury. Furthermore, high concentrations of NO are directly cytotoxic [33]. We have reported previously that the procoagulant proteins PDGF, which is released from the α-granules of activated platelets, and thrombin inhibit NO production, whereas the fibrinolytic enzyme plasmin enhances NO production from smooth muscle cells exposed to IL-1β [21–24]. The present study extends these findings by showing that these hemostatic proteins modulated NO synthesis by regulating the expression of the iNOS gene. In particular, the procoagulant proteins PDGF and thrombin inhibited NO release by inhibiting iNOS gene expression. Their inhibitory effects on iNOS mRNA levels were concentration dependent and paralleled their inhibitory action on nitrite synthesis. Plasmin also regulated smooth muscle cell NO synthesis by modulating the expression of iNOS mRNA. In contrast to PDGF and thrombin, however, plasmin treatment resulted in the potentiation, rather than inhibition, of IL-1β-stimulated expression of iNOS mRNA and nitrite release. Finally, these hemostatic proteins appeared to regulate IL-1β-stimulated iNOS mRNA levels at the level of transcription since co-stimulation with the various hemostatic proteins did not change the half-life of iNOS mRNA. Thus, these specific hemostatic proteins may regulate some step(s) in the signaling pathway of IL-1β to induce iNOS gene expression, or they may directly modulate the transcription of the gene itself.

Although these experiments were performed on vascular smooth muscle cells derived from the rat aorta, our laboratory and others have also demonstrated IL-1β-stimulated expression of iNOS activity in human vascular smooth muscle cells

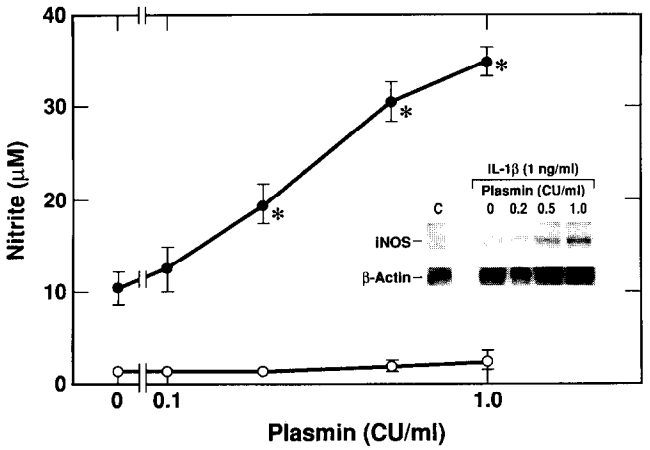

FIG. 7. Effect of plasmin on smooth muscle cell iNOS expression following IL-1β exposure. Nitrite production was measured from unstimulated (○) and IL-1β (1.0 ng/mL for 24 hr) stimulated (●) cultured rat aortic smooth muscle cells in the presence of plasmin (0.1 to 1.0 CU/mL). Results are the means ± SEM of four separate experiments performed in duplicate. Key: (*) statistically significant effect ($P < 0.05$) of plasmin treatment on IL-1β-stimulated nitrite release. Inset: Ribonuclease protection analysis of iNOS and β-actin mRNA from unstimulated (C) and from IL-1β (1.0 ng/mL for 4 hr) stimulated rat aortic smooth muscle cells in the presence and absence of plasmin (0.2 to 1.0 CU/mL). Similar findings were observed in four separate experiments.

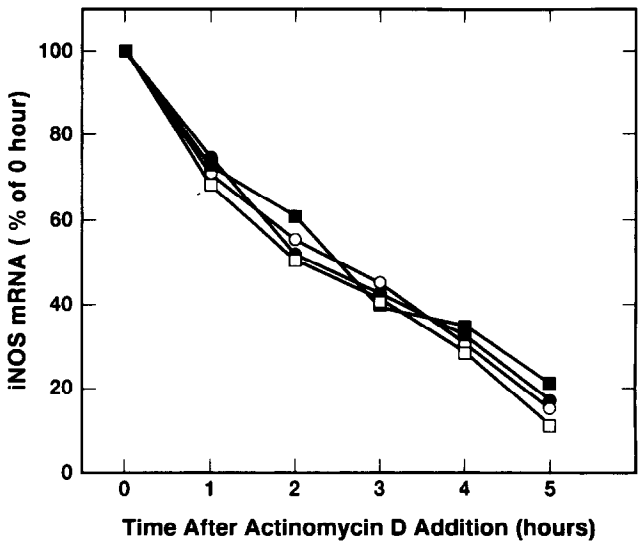

FIG. 8. Effect of hemostatic proteins on smooth muscle cell iNOS mRNA half-life following IL-1β exposure. Cultured rat aortic smooth muscle cells were stimulated with IL-1β (2.0 ng/mL for 4 hr) in the presence of vehicle (○), platelet derived growth factor (●), thrombin (□), or plasmin (■), and total RNA was extracted from the cells at the indicated times following actinomycin D (2 μg/mL) administration. To correct for differences in loading, the signal density of each RNA sample hybridized to the iNOS probe was divided by that hybridized to the β-actin probe. The corrected density was then plotted as a percentage of the 0-hr value against time. Similar findings were observed in three separate experiments.

[34, 35]. Furthermore, we have shown that the induction of iNOS activity in human vascular cells is similarly regulated by these hemostatic proteins [23]. Thus, the capacity of these hemostatic proteins to modulate iNOS gene expression in vascular smooth muscle may play a (patho)physiologically relevant role in humans.

In conclusion, the present study demonstrates that specific hemostatic proteins generated at sites of vascular injury regulated the expression of iNOS mRNA in vascular smooth muscle cells. PDGF and thrombin down-regulated the IL-1β-induced expression of iNOS, whereas plasmin up-regulated iNOS expression. The ability of components of the hemostatic system to suppress or amplify cytokine-induced expression of iNOS in vascular smooth muscle may provide an important mechanism by which the release of NO is regulated under certain pathological conditions.

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