Juxtacrine effects of IL-1α precursor promote iNOS expression in vascular smooth muscle cells

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Sasu, Sebastian, Angela L. Cooper, and Debbie Beasley. Juxtacrine effects of IL-1α precursor promote iNOS expression in vascular smooth muscle cells. Am J Physiol Heart Circ Physiol 280: H1615–H1623, 2001.—After injury to the blood vessel wall, vascular smooth muscle cells (SMC) synthesize interleukin (IL)-1 and inducible nitric oxide (NO) synthase (iNOS). The present study tested whether endogenous production of IL-1α stimulates iNOS expression in vascular SMC, and assessed whether IL-1α exerts autocrine effects on the cells producing IL-1α or juxtacrine effects on cells that contact the IL-1α producing cells. Rat aortic SMC were transiently transfected with expression plasmids encoding either IL-1α precursor, which localizes to the plasma membrane, or mature IL-1α, which remains cytosolic. iNOS mRNA levels, determined by RT-PCR, and production of nitrite, a stable oxidation product of NO, were markedly elevated in SMC overexpressing IL-1α precursor, and modestly elevated in SMC overexpressing mature IL-1α, relative to SMC transfected with vector alone. Exposure to exogenous IL-1β or TNF-α further stimulated iNOS gene expression in SMC producing IL-1α; low levels of IL-1β (20 pg/ml) were effective in SMC transfected with IL-1α precursor plasmid, whereas SMC transfected with mature IL-1α plasmid or vector alone required higher concentrations of IL-1β (200 and 2,000 pg/ml, respectively). The increases in iNOS mRNA levels and NO production in SMC overexpressing IL-1α precursor were prevented by exogenous IL-1 receptor antagonist, suggesting that these effects were mediated by the type I IL-1 receptor. Immunostaining studies indicated that IL-1α precursor stimulates iNOS gene expression via cell-cell contact. Expression of iNOS was enhanced in cells that were in contact with a cell overexpressing IL-1α precursor (identified by coexpression of green fluorescent protein), and in cells that were overexpressing IL-1α themselves, but only when the cell contacted another cell. Together these results indicate that IL-1α precursor acts by cell-cell contact as an autocrine and juxtacrine enhancer of iNOS gene expression, inducing moderate iNOS expression on its own, and markedly augmenting the responsiveness of rat aortic SMC to exogenous cytokines.

interleukin-1 receptor antagonist; tumor necrosis factor-α

INTIMAL HYPERPLASIA is a major clinical problem that limits the long-term efficacy of vascular procedures, such as balloon angioplasty and coronary bypass grafts, and a key component of this process is excessive vascular smooth muscle cell (SMC) proliferation (1, 38). The proliferative response evoked by vascular wall injury is preceded by a cascade of events, including synthesis of inducible nitric oxide (NO) synthase (iNOS), which likely modulates the extent of subsequent SMC proliferation. iNOS, like the constitutively expressed endothelial NOS (eNOS), produces NO via the five-electron oxidation of one of the guanidine nitrogens in L-arginine. However, distinct from eNOS, which is expressed in normal blood vessels and inhibits intimal proliferation evoked by vascular injury (34), it is not clear whether iNOS plays a pro- or antiproliferative role when expressed in injured blood vessels. NO, when added as a chemical NO donor or as NO gas, inhibits proliferation of SMC in vitro (18, 36, 33). However, NO has also been reported to stimulate SMC proliferation in vitro (20, 42), and neointima formation induced by mechanical injury is attenuated in iNOS knockout mice (13), suggesting iNOS may play a pro-proliferative role in this model.

Pro-inflammatory cytokines are the primary inducers of iNOS expression in many cell types, including rat aortic SMC. Among those that induce iNOS expression in rat aortic SMC in vitro, interleukin-1 (IL-1) is the most potent (6). Several lines of evidence suggest that SMC-derived IL-1 may be an important stimulus to iNOS expression in SMC. Vascular SMC synthesize both α- and β-forms of IL-1 when activated in vitro (7, 27, 44), and IL-1α is also synthesized by smooth musclelike cells in clinically relevant intimal hyperplastic lesions. IL-1α was detected by immunostaining in spindle-shaped cells of saphenous vein bypass grafts that had become stenotic, but not in internal mammary arteries that had remained patent, or in normal arteries and veins (12). Also, a recent study (5) indicates that low levels of IL-1α are biologically active when produced endogenously by human vascular SMC. IL-1α is synthesized as a 271-amino acid precursor molecule, which lacks a classical signal sequence (15), and is not efficiently released by cells, but nevertheless appears to be active in its cell-associated form. In several cell types, including SMC, IL-1α precursor is thought to associate with the plasma membrane in a
form that can activate the type I IL-1 receptor on adjacent cells via juxtacrime mechanisms (2, 22, 28). Recent studies have suggested that the IL-1α precursor may also act within the cell, by a mechanism involving direct localization to the nucleus (21, 30, 32, 46).

The present study determined whether endogenous production of IL-1α can induce iNOS gene expression in rat aortic SMC. Rat aortic SMC were transiently transfected with expression plasmids, which direct the production of either the precursor or mature form of IL-1α, and basal and inducible expression of iNOS was assessed. Because mature IL-1α lacks the nuclear localization sequence, and putative membrane localization signals that are present in the precursor molecule, and does not localize to the nucleus (5, 30) or associate with the plasma membrane (9, 11, 17), its expression should reveal the effects that occur after cellular release. To distinguish between autocrine versus juxtacrime actions of IL-1α precursor, immunostaining was used to separately assess iNOS expression in transfected cells themselves, and in those cells that contact a transfected cell. The results indicate that IL-1α precursor is an effective stimulus for iNOS gene expression in rat aortic SMC and can also augment iNOS expression induced by exogenous cytokines. In addition, juxtacrime effects appear to play a key role in the biological activity of IL-1α precursor.

METHODS

Rat aortic SMC culture. SMC were isolated from rat thoracic aorta by enzymatic dissociation as described previously (8). SMC were cultured in growth media composed of DMEM supplemented with 10% FCS, glutamine, penicillin, and streptomycin. Cells were passaged by harvesting with trypsin-EDTA and used between passages 3 and 12. Rat aortic SMC expressed α-actin, as determined by immunofluorescent staining using mouse anti-α-actin-FITC conjugate (Sigma).

IL-1α expression plasmids. IL-1α expression plasmids were constructed by PCR amplification of a plasmid containing human IL-1α cDNA, cloned from LPS-activated human peripheral blood cells (ATCC), as previously described (5), and cloned into pcDNA3. The IL-1α precursor expression plasmid encodes amino acids 1–271, whereas the mature IL-1α expression plasmid encodes amino acids 113–271, both cloned downstream of an introduced Kozak consensus sequence.

Transfection of rat aortic SMC by electroporation. Rat aortic SMC (5 × 10⁵/condition) were transfected by electroporation with pcDNA3 alone or encoding IL-1α precursor or mature IL-1α at 300 V and 960 μF (GenePulser, Bio-Rad). Cells were then plated and incubated overnight in media supplemented with sodium butyrate, 24 h in fresh media alone, and an additional 24 h in media with or without IL-1β (2 ng/ml). iNOS protein was localized in SMC coccultures by indirect immunofluorescence staining. Cells were washed in PBS, fixed in 3.7% formaldehyde (15 min), and permeabilized in 0.2% Triton X-100/PBS (5 min). Nonspecific binding sites were blocked with 10% normal horse serum, and the cells were incubated for 1 h at 37°C with polyclonal rabbit antisemur specific for murine macrophage iNOS (Cayman Chemical; Ann Arbor, MI) and then 45 min at room temperature with Texas-Red-coupled donkey anti-rabbit IgG (Jackson Immunoresearch). Cells were then incubated with Hoechst 33342 to stain cell nuclei, mounted in 90% glycerol/PBS, and observed through a microscope (Diaphot-TMD, Nikon) equipped with epifluorescence. The presence of iNOS was evaluated in transfected cells (GFP positive), in nontransfected cells (GFP negative) that were in direct contact with a transfected cell, and in cells that were neither transfected nor in direct contact with any other cell. For each treatment group, 300 cells (100 of each subgroup) were analyzed. The
observer performing the analysis was blinded with respect to the specimen treatment.

Statistical analysis. The significance of treatment-induced differences was determined either by Student's t-test or by ANOVA, followed by Dunnett's procedure to compare multiple means with a similar control value. \( P < 0.05 \) was considered statistically significant.

RESULTS

iNOS gene expression is enhanced in rat aortic SMC transfected with IL-1\( \alpha \) expression plasmids. Transient overexpression of either IL-1\( \alpha \) precursor or mature IL-1\( \alpha \) induced iNOS gene expression in rat aortic SMC. iNOS mRNA was not detectable by RT-PCR analysis in SMC transfected with vector alone, either 72 or 96 h after transfection (Fig. 1A). In contrast, iNOS mRNA was present in SMC that had been transfected with either IL-1\( \alpha \) precursor or mature IL-1\( \alpha \) expression plasmids. At both time points, the levels of iNOS mRNA were greater in SMC overexpressing IL-1\( \alpha \) precursor compared with SMC overexpressing mature IL-1\( \alpha \). To assess whether increased levels of iNOS mRNA were associated with increased iNOS activity, the levels of nitrite, a stable NO oxidation product, were measured in the supernatants, which overlayed the cells from 72–96 h after transfection. Nitrite production was significantly elevated in rat aortic SMC overexpressing either form of IL-1\( \alpha \) relative to those transfected with vector alone, and SMC overexpressing IL-1\( \alpha \) precursor produced more nitrite than SMC overexpressing mature IL-1\( \alpha \) (Fig. 1B). The levels of nitrite produced by SMC overexpressing IL-1\( \alpha \) precursor were low, however, accumulating to only 2–3 \( \mu M \) in 24 h relative to levels of 20–35 \( \mu M \), which are reached after stimulation with exogenous IL-1\( \beta \) (6). Nitrite production was also increased in SMC that were stably transfected with IL-1\( \alpha \) precursor expression plasmid (4.45 ± 1.32 \( \mu M/24 \) h per 10\(^5\) cells) relative to SMC stably transfected with vector alone (0.81 ± 0.29 \( \mu M/24 \) h per 10\(^5\) cells; \( P < 0.05 \)).

Expression of iNOS was consistently greater in SMC overexpressing IL-1\( \alpha \) precursor, relative to SMC overexpressing mature IL-1\( \alpha \). The enhanced efficacy of IL-1\( \alpha \) precursor relative to mature IL-1\( \alpha \) was not due to higher expression levels after transfection. In a previous study (5), IL-1\( \alpha \) precursor and mature IL-1\( \alpha \) were expressed at similar levels after transient transfection of a rat aortic SMC line with the corresponding expression plasmid, as indicated by Western blot analysis. Also, in the present study, rat aortic SMC transfected with IL-1\( \alpha \) precursor expression plasmid produced less IL-1\( \alpha \) (231 ± 51 pg/10\(^5\) cells) relative to those transfected with mature IL-1\( \alpha \) expression plasmid (475 ± 74 pg/10\(^5\) cells), as determined by a specific immunoassay. Thus the greater efficacy of the IL-1\( \alpha \) precursor expression plasmid was not due to higher expression levels.

Exogenous IL-1 receptor antagonist abolishes induction of iNOS in rat aortic SMC overexpressing IL-1\( \alpha \) precursor. The greater efficacy of IL-1\( \alpha \) precursor, compared with mature IL-1\( \alpha \), may be attributable to localization of the precursor molecule to the nucleus or to the plasma membrane, properties that are not shared by the mature IL-1\( \alpha \) molecule. Effects of membrane-associated IL-1\( \alpha \) precursor are mediated by the type I IL-1 receptor and are inhibited by high concentrations of exogenous IL-1 receptor antagonist (IL-1RA; 22), in contrast to the direct nuclear actions of IL-1\( \alpha \), which are thought to occur independently of this receptor (30, 32). To distinguish between these two mechanisms, the ability of exogenous IL-1RA to attenuate iNOS induction in rat aortic SMC overexpressing IL-1\( \alpha \) precursor was assessed. Addition of exogenous IL-1RA (10 \( \mu g/ml \)) compared with mature IL-1\( \alpha \), may be attributable to localization of the precursor molecule to the nucleus or to the plasma membrane, properties that are not shared by the mature IL-1\( \alpha \) molecule. Effects of membrane-associated IL-1\( \alpha \) precursor are mediated by the type I IL-1 receptor and are inhibited by high concentrations of exogenous IL-1 receptor antagonist (IL-1RA; 22), in contrast to the direct nuclear actions of IL-1\( \alpha \), which are thought to occur independently of this receptor (30, 32). To distinguish between these two mechanisms, the ability of exogenous IL-1RA to attenuate iNOS induction in rat aortic SMC overexpressing IL-1\( \alpha \) precursor was assessed. Addition of exogenous IL-1RA (10 \( \mu g/ml \))
to the cell culture media immediately after transfection prevented the subsequent induction of iNOS by expression of IL-1α precursor. iNOS mRNA was not detectable by RT-PCR in SMC overexpressing IL-1α precursor and incubated in the presence of exogenous IL-1RA, whereas iNOS PCR product was apparent in SMC overexpressing IL-1α precursor and incubated without IL-1RA (Fig. 2A). Also, nitrite production was not enhanced in SMC overexpressing the IL-1α precursor that were incubated in the presence of IL-1RA but was significantly enhanced in SMC overexpressing IL-1α precursor incubated in the absence of IL-1RA (Fig. 2B). These results indicate that IL-1α precursor acts extracellularly to stimulate iNOS gene expression via activation of the type I IL-1 receptor.

Increased sensitivity to exogenous IL-1β and TNF-α in rat aortic SMC overexpressing IL-1α. Exogenous IL-1β and TNF-α can act synergistically to induce iNOS expression in rat aortic SMC (6). Therefore, the ability of IL-1α produced by rat aortic SMC to act synergistically with exogenous IL-1β or TNF-α and induce iNOS activity was assessed. Exposure to exogenous IL-1β induced NO production in nontransfected and pcDNA3-transfected rat aortic SMC; however, a high concentration of IL-1β (2,000 pg/ml) was required to significantly stimulate nitrite production in both groups (Fig. 3A), as previously described (6). SMC transfected with IL-1α precursor expression plasmids produced significant nitrite in the absence of exogenous cytokine stimulation, as described above (Figs. 1B and 2B), and nitrite production was further enhanced by exposure to exogenous IL-1β, even at concentrations as low as 20 pg/ml. Although SMC overexpressing mature IL-1α did not produce nitrite in the absence of exogenous IL-1β stimulation, sensitivity to exogenous IL-1β was enhanced relative to SMC transfected with vector alone. SMC overexpressing mature IL-1α were not as sensitive to exogenous IL-1β as cells that overexpressed IL-1α precursor; significant stimulation of nitrite production required exposure to 200 pg/ml IL-1β, 10-fold more exogenous IL-1β than that required to stimulate nitrite production in rat aortic SMC overexpressing IL-1α precursor.
Exposure to high concentrations of exogenous TNF-α (50 ng/ml) did not induce significant NO production in either nontransfected or pcDNA3-transfected SMC (Fig. 3B). However, exogenous TNF-α (0.5–50 ng/ml) stimulated nitrite production in a concentration-dependent manner in SMC overexpressing either form of IL-1α. SMC overexpressing IL-1α precursor produced more nitrite than SMC overexpressing mature IL-1α at each concentration of TNF-α that was tested.

**IL-1α precursor stimulates iNOS expression in rat aortic SMC via juxtacrine effects.** Studies with IL-1RA indicated that the action of IL-1α precursor was extracellular and involved the type I IL-1 receptor. To assess the role of juxtacrine effects in the action of IL-1α precursor, immunostaining studies were conducted to localize iNOS expression in cocultures of SMC, which contained a mixture of transfected and nontransfected SMC. Rat aortic SMC were cotransfected with an IL-1α expression plasmid and an expression plasmid encoding GFP, as a marker of transfected cells. In preliminary studies, iNOS protein was not detectable by indirect immunofluorescence staining in SMC overexpressing IL-1α precursor in the absence of stimulation with exogenous cytokine, even though the cells expressed detectable iNOS mRNA and produced detectable nitrite (Figs. 1–3). The absence of detectable iNOS protein may be due to a lower sensitivity of the immunostaining procedure compared with the higher sensitivities of the RT-PCR and nitrite assays. This hypothesis was supported by subsequent experiments, in which iNOS protein was detectable in SMC exposed to exogenous IL-1β, a stimulus that induces higher levels of nitrite production than overexpression of IL-1α precursor (6). Surprisingly, however, <10% of nontransfected SMC expressed detectable iNOS protein after exposure to exogenous IL-1β (1 ng/ml), indicating an inherent variability of rat aortic SMC subpopulations in their sensitivity to exogenous IL-1β.

To analyze the spatial distribution of exogenous IL-1β-induced iNOS expression relative to the location of transfected cells, iNOS expression was assessed by immunostaining and scored in three different subpopulations of cells within the rat aortic SMC cocultures: cells that were transfected themselves (GFP positive), cells that were not transfected (GFP negative) but were in direct contact with a transfected cell, and nontransfected cells that were not in direct contact with any other cell (Fig 4). Immunoreactive iNOS was detectable in a small percentage of cells (>10%) within the pcDNA3-transfected rat aortic SMC cocultures, and this percentage was similar in all three subgroups: transfected cells, nontransfected cells that contacted a transfected cell, and nontransfected cells that were isolated from any other cell. The percentage of iNOS-positive cells was also low in all three subgroups of rat aortic SMC cultures, which contained cells overexpressing mature IL-1α. In contrast, the percentage of iNOS-positive cells was significantly greater in rat aortic SMC cocultures that contained cells overexpressing IL-1α precursor (GFP) expression in cocultures containing SMC co-transfected with IL-1α precursor and GFP expression plasmids.
Mature IL-1α, as described in Fig. 4. Percentage of cells expressing a direct cell-cell contact (10). IL-1α membrane-anchored ligands can also activate receptors and induce signals that influence cell function is well established.

Factors to activate membrane receptors and induce tissues are controlled by several forms of intercellular precursor expression plasmids. Expression with IL-1α and either pCDNA3 alone or pCDNA3 encoding IL-1α aortic SMC subpopulations. SMC were cotransfected with pEGFP H1620 JUXTACRINE IL-1α SMC transfected with IL-1α (juxtacrine), and nontransfected cells that did not contact any other cell were analyzed (4 and 10% respectively, iNOS positive). In contrast, iNOS expression was not enhanced in rat aortic SMC transfected with IL-1α precursor expression plasmid relative to rat aortic SMC transfected with vector alone when transfected cells that were isolated were analyzed (4 and 10%, respectively, iNOS positive).

**DISCUSSION**

The proliferation and function of cells in animal tissues are controlled by several forms of intercellular communication. The ability of diffusible polypeptide factors to activate membrane receptors and induce signals that influence cell function is well established. Membrane-anchored ligands can also activate receptors; however, this form of communication requires direct cell-cell contact (10). IL-1α precursor is one example of a polypeptide factor that has been proposed to act as a membrane-anchored ligand that activates cells via cell-cell contact (2, 17, 22, 25, 28). The present studies have three principal findings. First, they indicate that endogenous production of IL-1α precursor, as a sole stimulus, can induce iNOS gene expression and NO production in rat aortic SMC. Second, endogenous production of IL-1α precursor also sensitizes rat aortic SMC to the stimulatory effects of exogenous proinflammatory cytokines, including IL-1β and TNF-α. Finally, the primary mechanism of IL-1α precursor action involves juxtacrine effects, which are exerted via cell-cell contact, and involves activation of the type I IL-1 receptor.

Endogenous production of IL-1α precursor was a sufficient stimulus to induce detectable expression of iNOS in rat aortic SMC. Both iNOS mRNA and the release of nitrite, a stable NO oxidation product, were detectable in rat aortic SMC overexpressing IL-1α precursor either transiently or stably; however, the level of extracellular nitrite accumulation was low (~2 μM) relative to the nitrite levels found when rat aortic SMC are stimulated with exogenous IL-1b (~25 μM; Ref. 6). In previous studies (6), IL-1β and TNF-α, when added as soluble factors, acted synergistically to induce iNOS gene expression. In the present study, low levels of IL-1β and TNF-α (pg/ml) stimulated NO production in rat aortic SMC, which were producing IL-1α precursor. In contrast, higher levels of exogenous IL-1β (ng/ml) were required to induce NO production in the absence of endogenous IL-1α synthesis, and high concentrations of TNF-α were not effective. These results indicate that IL-1α precursor that is produced endogenously by rat aortic SMC can synergize with exogenous IL-1β or TNF-α to induce iNOS gene expression in SMC.

One mechanism by which IL-1α could exert local effects on gene expression is by acting as a soluble mediator after release from the cell. IL-1α precursor and mature IL-1α are equipotent agonists of the type I IL-1 receptor when presented to IL-1 receptor expressing cells as soluble factors (35). Also, both the precursor and mature forms of IL-1α can be released from cells (26, 39, 45), although both forms lack a signal sequence for secretion via classical pathways, and the secretory mechanism for both proteins is unknown. However, mature IL-1α appears to be the preferentially released form, on the basis of studies with human monocytes and bladder carcinoma cells (39, 45). Therefore, if IL-1α acted as a soluble extracellular factor after release from rat aortic SMC, then one would predict that overexpression of mature IL-1α would induce iNOS gene expression more effectively than overexpression of IL-1α precursor. In contrast, in the present studies overexpression of IL-1α precursor consistently induced higher levels of iNOS gene expression than overexpression of mature IL-1α. Therefore, the enhanced effectiveness of IL-1α precursor observed in this study argues against a primary mechanism involving cellular release of soluble IL-1α.

A second mechanism by which IL-1α could induce iNOS gene expression in rat aortic SMC is as a membrane-associated ligand. IL-1α precursor localizes to the plasma membrane after synthesis in LPS-stimulated cells (14, 41, 49), where it remains associated with the cell surface and is thought to activate the type I IL-1 receptor on adjacent cells via juxtacrine mechanisms (2, 17, 22, 25, 28). In contrast, mature IL-1α does not associate with the cell surface (9, 11, 17). In the present study, addition of IL-1RA, a competitive antagonist of IL-1 binding to the type I IL-1 receptor (31), abolished expression of iNOS in SMC transfected with IL-1α precursor expression plasmids. These results support an extracellular site of action involving the type I IL-1 receptor. Immunostaining studies also supported the hypothesis that IL-1α associates with the
surface of rat aortic SMC in a form that can activate type I IL-1 receptors via cell-to-cell contact. Cells in contact with IL-1α-precursor overexpressing cells demonstrated induction of iNOS, whereas isolated cells did not, indicating an important role of juxtacrine stimulation. iNOS expression was also enhanced in those SMC that were transfected with IL-1α precursor expression plasmids, however, only when the cell was in direct contact with another cell. It is possible that this effect involves two-way communication between SMC, which are in contact with each other. Because IL-1 is known to induce its own synthesis (43), membrane expression of IL-1α in the transfected cell could induce membrane expression of IL-1α in an adjacent cell, which in turn induces expression of iNOS in the transfected cell. Alternatively, it is possible that binding of IL-1α precursor to the type I IL-1 receptor on the adjacent cell elicits signal transduction events in the IL-1α precursor expressing cells as well as in the adjacent cell. This possibility has been proposed for juxtacrine mediators, which contain transmembrane domains (10). However, IL-1α precursor lacks a transmembrane domain, thus a mechanism by which IL-1α binding to its receptor could influence the IL-1α precursor expressing cell is not clear.

IL-1α precursor has also been proposed to act within the cell in some cell types, via a mechanism that is independent of the type I IL-1 receptor and involves direct localization to the nucleus (21, 30, 32, 46). In human vascular SMC and endothelial cells transfected with the corresponding expression plasmid, IL-1α precursor localizes to the nucleus, whereas mature IL-1α remains in the cytosol (5, 30, 46). The action of IL-1α precursor was linked to its nuclear localization in two studies with human endothelial cell lines. In one study, stable expression of IL-1α precursor stimulated expression of IL-1-inducible genes and inhibited cellular proliferation, whereas stable expression of mature IL-1α was ineffective (30). In the second study, stable expression of IL-1α precursor inhibited cell migration, whereas stable expression of mature IL-1α, or a nuclear localization deficient mutant of IL-1α precursor, stimulated cell migration (32). In both studies, the fact that exogenous IL-1RA did not reverse the effects of IL-1α precursor expression was used as evidence that IL-1α precursor acts within cells. In the present study, exogenous IL-1 receptor antagonist abolished iNOS expression in rat aortic SMC overexpressing IL-1α precursor, arguing against a role of nuclear localization in IL-1α precursor induced iNOS gene expression. The ineffectiveness of IL-1RA in previous studies could be due to the fact that lower IL-1RA concentrations were used (100 ng/ml). The present study used 10 μg/ml IL-1RA on the basis of evidence that higher concentrations are required to inhibit membrane-associated IL-1α activity (22). Alternatively, the short incubation with exogenous IL-1RA (20–24 h) may not have been sufficient time to reverse the long-term effects in stable transfectants that had been producing IL-1α precursor for many weeks. In support of this interpretation, the mitogenic effects of long term IL-1 exposure in human SMC are only partially reversed by a 72-h exposure to IL-1RA (5). It is possible that localization to the plasma membrane, rather than to the nucleus, also accounts for the actions of IL-1α precursor in human endothelial cells (30, 32).

Several lines of evidence indicate that SMC-derived iNOS contributes to the modulation of SMC proliferation in pathophysiological states. However, iNOS may limit or promote intimal hyperplasia, depending on the pathophysiological condition involved. Vascular SMC within balloon-injured rat carotid arteries express iNOS (47), and targeted deletion of the iNOS gene in mice is associated with a decrease in neointimal thickening after mechanical injury to the carotid artery (13), consistent with a pro-proliferative role of iNOS. In distinct contrast, other studies indicate that iNOS limits SMC proliferation, which occurs in coronary vessels after cardiac transplantation. iNOS is expressed by SMC within coronary arteries of transplanted human hearts that exhibit accelerated graft arteriosclerosis (37). Also, the accelerated arteriosclerosis that occurs after allogeneic cardiac transplantation is exacerbated in iNOS-deficient mice (24), indicating a protective role of the enzyme in limiting excessive SMC proliferation in the setting of transplant rejection. iNOS is also expressed by SMC within atherosclerotic lesions of the human aorta (29). However, whether iNOS plays a protective or deleterious role in atherosclerosis remains to be established.

Expression of iNOS in vascular SMC has also been proposed to contribute to vasodilatation that occurs during sepsis (8). Exposure of intact rat aortic segments to bacterial lipopolysaccharide (LPS) or IL-1 in vitro markedly inhibits vascular contraction, an effect that is independent of the endothelium and involves expression of iNOS (3, 4, 6, 16). iNOS is also expressed in rat aorta after injection of bacterial LPS in vivo (6); however, immunostaining studies indicated that iNOS protein is expressed primarily in adventitial fibroblasts, whereas iNOS expression was not detectable in medial SMC (48). The adventitia may also be the primary source of NO in intact rat aortic segments exposed to LPS in vitro, on the basis of evidence that intact rat aortic segments produce more NO after LPS stimulation than do segments of rat aortic media from which the adventitia was removed (23). In the present study, rat aortic SMC were obtained by enzymatic digestion of the aortic media after separation from the adventitia, and a subpopulation of cells that subsequently grew in culture expressed high levels of iNOS protein after stimulation with exogenous IL-1β. Another subpopulation of rat aortic SMC showed high levels of iNOS protein when activated by two stimuli, soluble IL-1β and membrane-associated IL-1α precursor. SMC derived from enzymatic digestion of the media of many blood vessels, including the rat aorta (40), are known to be heterogeneous with respect to phenotype. Another aspect of this heterogeneity may be the endogenous production of IL-1α, or alternatively IL-1β or TNF-α. The present study indicates that differential endogenous production of IL-1α in rat aortic SMC
subpopulations may account for differential sensitivity to exogenous cytokines.

The present study provides evidence that endogenous production of IL-1α precursor can induce low-level iNOS gene expression in rat aortic SMC and can act synergistically with exogenous IL-1β and TNF-α to induce high-level iNOS expression. A primary mechanism of IL-1α precursor action involves juxtacrine effects that are exerted via cell-cell contact and are mediated via activation of the type I IL-1 receptor. SMC-derived IL-1α precursor may be a clinically relevant stimulus to iNOS expression in SMC and may contribute to the development of intimal hyperplastic lesions in injured blood vessels or transplanted hearts.

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