Osteopontin inhibits inducible nitric oxide synthase activity in rat vascular tissue

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Osteopontin inhibits inducible nitric oxide synthase activity in rat vascular tissue. Am. J. Physiol. 275 (Heart Circ. Physiol. 44): H2258–H2265, 1998.—We tested the hypothesis that osteopontin (OPN) can inhibit the induction of inducible nitric oxide synthase (iNOS) in vascular tissue. iNOS activity was induced in rat thoracic aortas by incubation of the tissue with lipopolysaccharide (LPS) and measured by conversion of L-[3H]arginine to L-[3H]citrulline. Addition of >1 nM recombinant OPN protein significantly reduced the LPS-induced increase in iNOS activity. Western blotting and the RT-PCR were used to determine the effect of LPS with and without OPN on tissue levels of iNOS protein and RNA, respectively. LPS resulted in an increase in iNOS protein and RNA, whereas OPN dose-dependently reduced tissue levels of iNOS activity, protein, and RNA. Mutated OPN proteins, in which the integrin-binding RGD amino acid sequence was deleted or mutated to RGE, resulted in complete and partial loss, respectively, of the ability of OPN to inhibit LPS-induced iNOS activity, implicating integrin binding in the effect. These results indicate that OPN can prevent induction of iNOS in vascular tissue.

lipopolysaccharide; sepsis; endotoxin

OSTEOPONTIN (OPN) is an integrin- and calcium-binding phosphoprotein produced by a limited set of normal cells, including cells of mineralized tissue, epithelial cells, and activated cells of the immune system (4, 31). OPN production has been reported to be increased in a number of pathological conditions, including inflammation, atherosclerosis, nephritis, and malignancy, as well as normal situations of morphogenesis, such as bone remodeling (10, 31, 39). OPN is expressed in various tissues of the vascular system in response to injury and appears to play a role in the maintenance of normal vascular development and function (9, 10, 12, 30). The function of OPN in the normal and pathological contexts in which it is expressed remains poorly understood. However, many of its effects appear to be mediated by interaction of OPN, via its conserved GRGDS (glycine-arginine-glycine-aspartic acid-serine) amino acid sequence, with integrin molecules (especially αvβ3) (21, 41, 42), and perhaps other receptors, on the surfaces of target cells.

OPN has been shown to inhibit the induction of the inducible nitric oxide synthase (iNOS) expression and function in kidney epithelial cells in culture (13), suggesting that OPN may play a role in regulating the nitric oxide (NO) synthetic pathway in the kidney. OPN has also been demonstrated to inhibit NO production and reduce cytotoxic activity in macrophages (35, 36). An OPN fragment has also been shown to modulate iNOS mRNA levels in microvascular endothelial cells from the heart (40). On the basis of these findings, coupled with the demonstration that OPN produced by tumor cells can contribute functionally to their malignancy (1, 8, 28) and that NO mediates the cytotoxic activity of macrophages (11), it has been hypothesized that OPN produced by tumor cells could function to protect them from NO-mediated cytotoxic attack by host vascular and immune tissues (3, 7).

We tested the hypothesis that OPN inhibits the induction of iNOS in vascular tissue. Rat thoracic aortas were treated with lipopolysaccharide (LPS) to induce iNOS activity, and the effect of added recombinant mouse OPN on iNOS and constitutive NO synthase (cNOS) activities was determined. OPN proteins lacking the RGD sequence (and, therefore, unable to bind to integrins) or with a substituted RGE (arginine-glycine-glutamic acid) sequence, which binds poorly to integrins, were also used to address the requirement for the RGD sequence in OPN inhibition of iNOS activity. Furthermore, Western blotting and RT-PCR were used to assess iNOS protein and mRNA levels, respectively, in the vascular tissue. These investigations provide evidence that OPN and NOS expression may be functionally linked in vascular tissues.

METHODS

Preparation of Recombinant OPN

Recombinant mouse OPN was expressed in Escherichia coli as a glutathione S-transferase (GST)-fusion protein and purified as described previously (42, 43). Mutated OPN proteins in which the RGD sequence was deleted (and thus unable to bind to integrins) or substituted with RGE (and thus poorly bound to integrins) were produced by site-directed mutagenesis, as described previously (43). GST protein was used as a control.

Incubation of Thoracic Aorta

Male Sprague-Dawley rats (280–350 g) were anesthetized with pentobarbital sodium (50 mg/kg ip) and killed by cervical dislocation and exsanguination. Thoracic aortas were flushed with Krebs solution (pH 7.4; in mM: 118 NaCl, 4.7 KCl, 2.5 CaCl2, 1.2 MgSO4·7H2O, 1.2 KH2PO4, 11 dextrose, 22
NaHCO₃), removed, and cleared of surrounding connective tissue. Each vessel was dissected into four equal segments, such that each rat contributed a segment of thoracic aorta to each of four groups for incubation. In each experimental protocol, vessels were incubated for 5 h at 37°C and aerated each of four groups for incubation. In each experimental tissue. Each vessel was dissected into four equal segments, nitrogen and maintained at 80°C until the time of assay. Pilot experiments documented no effect of the GST protein (to which the recombinant OPN is linked) on the LPS induction of iNOS activity.

To evaluate the concentration-dependent effect of OPN on induction of iNOS activity and expression, rat thoracic aortae were incubated with a previously determined concentration of LPS (1 µg/ml) in Krebs solution (38). The tissues were co-incubated with and without OPN (0.1–100 nM).

The effect of OPN on cNOS and iNOS activities and expression of iNOS in control and LPS-treated vascular tissues were assessed. Rat thoracic aortae were incubated with and without LPS (1 µg/ml) and with and without OPN (10 nM). This supramaximal concentration of OPN was selected on the basis of the results of the initial concentration-determination study.

To examine the role of integrin binding in the effect of OPN on LPS-induced iNOS activity and expression in stimulated rat vascular tissue, alternative forms of OPN, which had been mutated at the GRGDS sequence, were used. Rat aortae were incubated with LPS (1 µg/ml) under control conditions (no LPS) or with the intact recombinant OPN, the RGD-deleted form of OPN, or the RGE-mutated form of OPN (10 nM each).

NOS Assay

After homogenization in ice-cold homogenization buffer, NOS activity was quantitated in thoracic aortae as the conversion of L-[3H]arginine to L-[3H]citrulline, as previously described (38). The protein concentrations of rat aortic homogenates were determined by the Bradford method (2), with BSA as the standard and homogenization buffer as the blank.

Calcium-dependent (constitutive) NOS activity was calculated as the difference between a sample containing calcium and calmodulin (cNOS + iNOS activities) and one containing EDTA/EGTA (iNOS activity only). Nonspecific radioactivity and/or metabolism of L-arginine was accounted for by incubating homogenization buffer or tissue homogenate with 10 µM N⁶-nitro-L-arginine methyl ester (an inhibitor of the NOS-mediated conversion of L-arginine), respectively, in the incubation buffer containing EDTA/EGTA, as described previously (38).

Resultant enzyme activities were expressed as picomoles of L-[3H]citrulline produced per minute per milligram of protein.

Cell Culture

RAW 264.7 mouse macrophage cells (American Type Culture Collection, Rockville, MD) were grown at 37°C in 5% CO₂ in DMEM with 10% fetal bovine serum (GIBCO BRL, Burlington, ON, Canada). Cells grown to confluence were untreated (control) or treated with 20 ng/ml LPS plus 50 µg/ml murine interferon-γ (IFN-γ) for 6 h to induce iNOS.

Rat aortic smooth muscle cells were grown at 37°C in 5% CO₂ in DMEM with 10% calf serum and 5% fetal bovine serum. Cells grown to confluence were untreated (control) or treated with 250 µg/ml LPS plus 1,000 µg/ml murine IFN-γ for 10 h to induce iNOS.

Western Blots

Rat thoracic aortae were homogenized in five volumes of cold homogenization buffer (pH 7.4; 10 mM Tris, 1 mM EDTA, 2 µg/ml leupeptin, 1 µg/ml pepstatin, 0.5 mM phenylmethylsulfonyl fluoride). An aliquot of 20% SDS was added to give 1% final concentration, and samples were boiled for 5 min. Protein extracts were prepared from cell cultures (RAW 264.7 and rat aortic smooth muscle cells) by washing each culture dish twice with PBS (pH 7.5; in mM: 137 NaCl, 2.7 KCl, 8.15 Na₂HPO₄, 1.5 KH₂PO₄) and adding boiling buffer (pH 7.4; 10 mM Tris, 1% SDS, 2 µg/ml leupeptin, 1 µg/ml pepstatin, 0.5 mM phenylmethylsulfonyl fluoride). Each cell lysate was scraped from the dish, boiled 5 min, and passed through a 26-gauge needle. All samples were centrifuged at 16,000 g for 5 min to precipitate insoluble material, each supernatant was removed, and its protein concentration was determined by Peterson's modification (32) of the Lowry method, with BSA as the standard.

Proteins (5 µg each for RAW 264.7 macrophages and rat aortic smooth muscle cells; 25 µg for rat thoracic aortic homogenate) were separated by SDS-PAGE under reducing conditions on a 6% separating gel and electrophoretically transferred to Immobilon-P (Millipore, Bedford, MA). Blots were blocked with 4% casein in Tris-buffered saline (TBS; pH 7.4; 20 mM Tris, 150 mM NaCl) for 1 h at 37°C. Blots were then incubated overnight at room temperature in 4% casein in TBS with a mouse iNOS-specific monoclonal antibody (1:1,250), washed, and incubated for 3 h at room temperature in 4% casein in TBS, with an anti-mouse Ig secondary antibody linked to horseradish peroxidase (1:3,000). The iNOS band was detected by enhanced chemiluminescence protocol with Hyperfilm-ECL (Amersham, Oakville, ON, Canada). Results were quantitated by densitometry using a PhosphorImager SI (Molecular Dynamics, Sunnyvale, CA).

Primer Design

The rat iNOS cDNA sequence was obtained from GenBank (accession no. X76881): 5'-TGGGTGGCCCTTCTTGCTCCAAGGTTTGCTATAT-3' (forward primer, located at nt 1654–1678) and 5'-GTTGTGTTCTCTCTCCAGGTTTTGCTAT-3' (reverse primer, located at nt 1901–1930). This primer set is perfectly complementary to the rat iNOS and not complementary to other NOS isoforms or unrelated cDNAs. Amplification of cDNA synthesized from mRNA produces a 250-bp PCR band.

The mouse glyceraldehyde 3-phosphate dehydrogenase (GAPDH) sequence was obtained from GenBank (accession no. M32599): 5'-ATTGGGCCCTTGGTACCA-3' (forward primer, located at nt 79–98) and 5'-CCACCTTTGGAGTTTGCTAT-3' (reverse primer, located at nt 806–825). This primer set is perfectly matched to the rat and mouse GAPDH cDNA. A 752-bp PCR fragment will be synthesized. Oligonucleotide primers for iNOS and GAPDH were synthesized by the Medical Research Council Molecular Biology Core Facility at London Regional Cancer Centre.

RT-PCR Analysis

Reverse transcription was performed using the reverse oligonucleotide primers for rat iNOS and mouse GAPDH (a control reaction). One microgram of total cytoplasmic RNA isolated from each tissue using TRIzol reagent (GIBCO BRL) was reverse transcribed in a 20-µl reaction mixture according to the recommended conditions in the Moloney-murine leukemia virus RT kit (GIBCO BRL). A negative control of sterile water, in place of RNA, ensured no contamination of reaction materials.
The cDNAs (5 µl) produced by the RT reaction were then amplified by the PCR by using the forward oligonucleotide primers for iNOS and GAPDH. The PCR amplification was performed according to the manufacturer’s protocol (GIBCO BRL). RT-PCR conditions were optimized to ensure that the procedure was performed in the linear portion of the reaction. The RT-PCR products (5 µl) were identified by size on an ethidium bromide-stained 1.5% agarose gel. The gel was then denatured and neutralized, and the DNA capillary was transferred onto GeneScreen Plus (NEN/Du Pont, Mississauga, ON, Canada) in the presence of 10× saline-sodium citrate. The blot was probed sequentially with a 5’-end [32P]ATP-labeled iNOS oligonucleotide probe complementary to an internal rat iNOS-specific sequence (5’-TCAGTAGCAAGAGGACTGTGGCTCTGACG-3’, nt 1785–1814) and a denatured, oligo-labeled (deoxy-[32P]CTP) GAPDH cDNA probe (oligo-labeling kit, Pharmacia Biotech, Baie d’Urfé, PQ, Canada). The RT-PCR products were quantified by densitometry (PhosphorImager SI), and expression levels were calculated relative to GAPDH.

To confirm that the amplified PCR products represented iNOS mRNA, PCR cDNA products were directly ligated into the PCR II vector and transformed into bacteria (competent INVαF8 cells) by the TA cloning kit (Invitrogen, San Diego, CA). Selection was by blue-white screening and analysis by Miniprep to verify the presence of cloned PCR products. The products were then expanded and purified by plasmid preparation (Prep-a-Gene Kit, Bio-Rad, Mississauga, ON, Canada) and confirmed by direct sequencing using the T7 sequencing kit (Pharmacia Biotech).

**Chemicals**

Cytoscint environmentally safe scintillation fluid and Coo massie brilliant blue G-250 were purchased from ICN Biomedical (Mississauga, ON, Canada). Orthophosphoric acid was purchased from BDH (Toronto, ON, Canada). Murine monoclonal iNOS-specific antibody was purchased from Transduction Laboratories (Lexington, KY). TRIzol reagent, SDS, calf serum, DMEM, and recombinant murine IFN-γ were purchased from GIBCO BRL. Anti-mouse IgG antibody linked to horseradish peroxidase and L-[3H]arginine were purchased from Amersham. FCS was purchased from Whittaker Bioproducts (Walkersville, MD). E. coli LPS (serotype 026:B6) and all other reagents were purchased from Sigma Chemical (Mississauga, ON, Canada).

**Statistical Analysis**

All enzyme activity and densitometry results are expressed as means ± SE and compared using factorial ANOVA with Fisher’s post hoc test. *P* < 0.05 was considered significant. All statistical tests were calculated using the StatView + 4.5 program on a Macintosh computer.

**RESULTS**

**NOS Activity of Rat Thoracic Aorta**

Effect of OPN on control and LPS-stimulated iNOS and cNOS activities. To determine whether OPN affected iNOS and cNOS activities in LPS-stimulated and control tissues, vessels were incubated for 5 h with and without LPS (1 µg/ml) in the presence of OPN (0–10 nM). Tissues incubated with LPS alone exhibited an elevated iNOS activity compared with control incubated tissues (Fig. 1A). Incubation of aortas with LPS and OPN (0.1–10 nM) resulted in a progressive attenuation of the LPS-induced iNOS activity, producing a significant attenuation at ≥1 nM OPN.

A concomitant decrease in the cNOS activity of the LPS-incubated tissues was demonstrated relative to control tissues (Fig. 1B). cNOS activity in the vessels incubated with LPS and ≥1.0 nM OPN was similar to control and thus significantly greater than in vessels incubated with LPS alone. Incubation of control vessels with OPN (10 nM) alone did not result in any significant alteration of cNOS or iNOS activities compared with controls. Incubation of control and LPS-stimulated tissues with the GST protein had no significant effect on NOS activities (data not shown). We further confirmed that OPN had no effect on induced iNOS...
activity in tissues previously incubated for 5 h with LPS only (18.1 ± 2.7 vs. 22.1 ± 1.0 pmol l-citrulline·min⁻¹·mg⁻¹ for LPS-stimulated tissues with and without addition of 10 nM OPN at time of enzyme activity determination, respectively; n = 2 assays each).

Effect of RGD-mutated OPN on LPS-stimulated tissues. To determine whether the RGD amino acid sequence of OPN was required for the OPN blockade of LPS-induced changes in iNOS and cNOS activities, vessels were incubated with the RGD-deleted and RGE-mutated forms of OPN. In contrast to the effect seen with nonmutated OPN (Fig. 1), coincubation of thoracic aortas with LPS and the RGD-deleted form of OPN (10 nM) did not significantly alter the iNOS and cNOS activities compared with LPS-incubated tissues (n = 5 assays; Fig. 2). Coincubation of thoracic aortas with LPS and the RGE-mutated form of OPN (10 nM) significantly attenuated the enhanced iNOS activity observed with LPS-treated tissues (n = 5 assays). cNOS activity was also increased in the tissues incubated with LPS and RGE-mutated OPN compared with LPS-incubated tissues (n = 5 assays), although not to the level of cNOS activity observed in control tissues.

**iNOS Protein Levels in Rat Thoracic Aorta**

As OPN blocked the induction of iNOS activity by LPS (Fig. 1) but was unable to directly inhibit previously induced iNOS activity, Western blotting was performed using an antibody specific for mouse macrophage iNOS, to examine whether the OPN effect was due to an inhibition of the upregulation of iNOS protein levels (Fig. 3). To verify the specificity of this antibody, iNOS protein amounts were examined in a mouse macrophage cell line (RAW 264.7) and in cultures of rat aortic smooth muscle cells, both of which were stimulated with LPS and IFN-γ to induce iNOS. Western blotting showed that this antibody detected an ~123-kDa iNOS protein in both types of stimulated cells that was not present in unstimulated cells (Fig. 3A). Hence, this antibody recognized mouse macrophage iNOS and rat vascular smooth muscle iNOS.

Analysis of rat aortic homogenates by Western blotting with this antibody showed an iNOS protein band that comigrated with those seen in stimulated RAW 264.7 cells and rat aortic smooth muscle cells, although the intensity of the band from the cell lines was considerably greater than that from the tissue homogenates. A nonspecific binding band (~133 kDa) was detected in all aortic tissue homogenate samples, even when the iNOS antibody was omitted and only the secondary antibody was used with aortic homogenate; this nonspecific band was not detected in the RAW 264.7 cell line.

iNOS protein levels in each sample were determined by densitometry and expressed relative to the LPS-stimulated maximum (Fig. 3B). iNOS protein levels in naive control and untreated aorta (control) were low and comparable to those seen in vessels exposed to OPN alone (NOS activities of these 2 groups were not statistically significant, data not shown). A slight elevation of iNOS protein in tissues incubated under control conditions (compared with naive control tissues) was likely because of low levels of LPS normally found on the glassware during the incubation (34). Addition of LPS (1 μg/ml) to the incubation medium resulted in a significant increase in aortic iNOS protein levels compared with control incubated tissues. The presence of LPS plus increasing doses of OPN slightly, although not significantly, attenuated iNOS protein levels relative to LPS treatment alone, but not to the same extent as with the iNOS enzyme activities. Coincubation of the tissues with LPS and the RGD-deleted or RGE-mutated OPN did not affect the iNOS protein levels (not significantly different from LPS treatment alone, n = 3 for each group; data not shown).

**iNOS RNA Levels in Rat Thoracic Aorta**

To determine whether the OPN-mediated inhibition of the LPS-induced iNOS activity was due to transcriptional regulation, iNOS RNA levels were examined by RT-PCR followed by Southern blotting and probing with an internal iNOS-specific oligonucleotide probe. Analysis of the RT-PCR products from naive control and LPS-stimulated tissues indicated significant induction of a 250-bp band that corresponded to the band induced in rat aortic smooth muscle cells stimulated with LPS and IFN-γ (Fig. 4). Consistent with the Western blot data (Fig. 3), this 250-bp iNOS band was progressively decreased (again, not significantly) in tissues coincubated with LPS and OPN (0.1–10 nM) and, again, not to the same extent observed with the iNOS activities.

**DISCUSSION**

The experiments presented here indicate that OPN can block the LPS-induced increase in iNOS activity and reduce the expression of iNOS observed in rat thoracic aorta and that this blockade is dependent on the concentration of OPN used. This effect was not due to direct inhibitory activity toward the iNOS enzyme, as the addition of OPN to previously LPS-stimulated...
tissues had no effect on iNOS activity. Furthermore, this investigation demonstrates for the first time that OPN blocks the LPS-induced depression of cNOS activity in the vasculature. Recombinant OPN protein from which the RGD region had been deleted to produce a protein that is unable to bind to cell surface integrins was ineffective at blocking the LPS-induced increase in iNOS. Mutated OPN in which the RGD sequence had been mutated to RGE and, therefore, binds poorly to integrins was only partially effective at blocking the LPS-induced elevation of iNOS activity. Thus the effect of OPN on the induction of iNOS in this tissue depends on an intact integrin-binding RGD amino acid sequence in the protein, consistent with an integrin-mediated signal transduction process. Our study demonstrates that OPN can modulate LPS-mediated alterations of iNOS and cNOS in vascular tissue.

We have previously demonstrated that stimulation of vascular tissues with LPS results in an increase in iNOS activity, along with a concomitant decrease in cNOS activity (38). In the present study we confirmed this finding and found, for the first time, that OPN coincubated with LPS was able to completely block the LPS-mediated effects on iNOS and cNOS activities. The decrease in cNOS activity, coordinated with an increase in iNOS activity, has been hypothesized to be mediated at the mRNA level through a reduction in the stability of the cNOS mRNA (22, 44). Although this study did not directly examine the mechanism of these observed changes in cNOS activity, our data would be consistent with this hypothesis and further suggest that OPN interferes with this induction/downregulation signal transduction pathway.

In the present study, we used Western blotting and RT-PCR to assess levels of iNOS protein and RNA, respectively, in LPS-stimulated vascular tissue, with and without OPN. We demonstrated that LPS increased tissue levels of iNOS protein and RNA, consistent with the observed increase in iNOS activity. Concomitant with the decrease in iNOS activity observed in tissues coincubated with LPS and OPN, iNOS protein and RNA levels were attenuated compared with tissue incubated with LPS alone, although not to control levels. These results are consistent with previ-
ously reported findings for kidney proximal tubule epithelial cells in culture (13), in which OPN reduced the cytokine-induced increase in cellular levels of iNOS protein, and with previously reported findings for cultured cardiac myocytes and endothelial cells, where exogenous application of an OPN fragment concomitantly reduced iNOS RNA expression and protein levels (40). However, the finding that the degree of inhibition of induction of iNOS protein and mRNA did not precisely correlate with the observed restoration to control levels of iNOS activity suggests that additional mechanisms of iNOS regulation exist. Recently, Eissa et al. (6) reported that in human epithelial cells an iNOS splice variant may be produced, which is unable to form the active iNOS dimer. Hence, some of the increased iNOS RNA and protein observed in our LPS-treated tissues may produce nonfunctional iNOS protein, and the observed reduction of RNA and protein might be sufficient to account for the more significant attenuation of iNOS activity. Thus we have demonstrated that OPN produces a unique inhibition of the LPS-stimulated induction of iNOS activity in the vasculature, which is mediated, at least in part, by regulation of iNOS RNA and protein levels.

We have demonstrated that the presence of the RGD amino acid sequence is essential for the OPN-mediated blockade of the LPS-induced elevation of iNOS activity. Although the exact mechanism of this inhibition remains unknown, the inability of the RGD-deleted OPN to prevent the LPS-mediated induction of iNOS activity and of the concomitant reduction in cNOS activity (demonstrated with normal OPN) implicates integrin binding, perhaps to αvβ3, and the resultant signal transduction pathway in this response. Furthermore, what appears to be a partial blockade of this iNOS effect with the RGE-mutated OPN suggests a partial agonist activity of this mutant form. This observation is consistent with the previous finding of Hwang et al. (13), who found that OPN was unable to block the induction of nitrite production in stimulated kidney epithelial tubule cells coincubated with GRGDS peptide fragments. Together, these investigations suggest that an integrin binding-mediated event stimulates the

Fig. 4. Effect of OPN on LPS induction of iNOS RNA in rat thoracic aortas. A: Southern blot of iNOS and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) RT-PCR products from stimulated (+) and unstimulated (−) RASMC and rat thoracic aortas from naive rats and vessels incubated with LPS (1 µg/ml) and 0–10 nM OPN from a representative incubation experiment, for which tissue was obtained for Western and Southern blotting and for NOS activity determination. B: quantification of iNOS RNA levels obtained from Southern blotting of RT-PCR products, normalized to RNA levels in LPS-stimulated tissues. Results are from quantification of Southern blot by PhosphorImager SI expressed as ratio of 250-bp iNOS band to 750-bp GAPDH band to control for variability. Values are means ± SE, with number of observations in parentheses above bars. *P < 0.05 compared with control or unstimulated tissues; #P < 0.05 compared with LPS-stimulated tissues.
signal transduction pathway (14, 15) necessary to produce the inhibitory effects of OPN on the LPS-stimulated alteration of iNOS and cNOS.

Both of the major cellular components of vascular tissue, namely, smooth muscle and endothelial cells, have been shown to be capable of producing and responding to OPN. Giachelli and co-workers (9, 10, 20, 21) identified OPN as a protein induced in vascular smooth muscle tissue undergoing morphological change and repair, as well as in proliferating smooth muscle cells, and it has been reported that quiescent vascular smooth muscle cells do not produce OPN in culture. Uninjured rat endothelium expresses low levels of OPN and one of its integrin receptors (αvβ3), and expression of OPN and αvβ3-integrin transiently increases during repair of vascular injury (21). Thus OPN can be produced by smooth muscle and endothelial cells under situations of repair and morphogenesis (9). Clearly, the interaction of these two cell types and their products (i.e., NO and OPN) will contribute to the functional response of vascular tissue to activating agents such as LPS.

Excess production of NO through the action of iNOS may be an important mechanism that contributes to the hypotension (25) and abnormal vascular contractility characteristic of sepsis (38). Furthermore, NO has significant effects on leukocyte adhesion and a variety of other physiological processes that may be important in conditions such as sepsis or inflammation. Corticosteroids have been demonstrated to inhibit the induction of iNOS that occurs in response to LPS (17, 33), and recently, Singh et al. (40) reported that this effect of corticosteroids in cardiac myocytes and endothelial cells may be mediated through OPN. These data, along with the results of the present investigation, suggest that OPN may be important in the regulation of iNOS activity in other tissues in conditions such as sepsis.

The results presented here are the first to demonstrate that OPN is capable of preventing the inducible increase in iNOS and decrease in cNOS activity in vascular tissue, suggesting that OPN plays a role in regulating NO activity in the vasculature. Because NO is a highly reactive and potentially toxic molecule, the production and activity of NO are likely to be tightly regulated (24, 26, 37). Our findings with RGD-mutated OPN proteins implicate integrin-mediated binding and likely signal transduction in the blockade of LPS-induced iNOS activity. One potential mechanism of the OPN blockade of the iNOS activity may be inhibition of the induction of iNOS mRNA and protein in a subset of vascular cells (i.e., endothelial and vascular smooth muscle cells). The results presented here suggest that OPN may be an important mediator of the physiological and pathophysiologic regulation of iNOS in vascular tissue in health and disease.

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