Glucocorticoids Increase Osteopontin Expression in Cardiac Myocytes and Microvascular Endothelial Cells

ROLE IN REGULATION OF INDUCIBLE NITRIC OXIDE SYNTHASE*

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In heart muscle, the cytokine-inducible isoform of nitric oxide synthase (NOS2) is expressed in both cardiac myocytes and microvascular endothelial cells (CMEC). mRNA levels for both NOS2 and for osteopontin, a multifunctional extracellular matrix phosphoprotein containing an RGD integrin binding domain, are increased in cardiac muscle following intraperitoneal injection of adult rats with lipopolysaccharide. In vitro, interleukin-1β and interferon-γ increased osteopontin mRNA levels in CMEC as well as NOS2 expression in both CMEC and cardiac myocytes. However, osteopontin mRNA levels in heart muscle in vivo, and in cardiac myocytes and CMEC in vitro, are also increased 10-30-fold by the synthetic glucocorticoid dexamethasone, an agent that suppresses cytokine induction of NOS2 in both cell types. The hexapeptide GRGDSP, which interrupts binding of RGD-containing proteins to cell surface integrins, increased NOS2 mRNA, while a synthetic osteopontin peptide analogue decreased NOS2 mRNA and protein levels in both cytokine-pretreated cardiac myocytes and CMEC cultures. Also, transfection with a full-length antisense-osteopontin cDNA in cytokine-pretreated CMEC decreased endogenous osteopontin mRNA and increased NOS2 mRNA levels. These results suggest that osteopontin could regulate the location and extent of NOS2 induction in the heart. Increased expression of osteopontin also may be one mechanism by which glucocorticoids suppress NOS2 activity in cardiac myocytes and microvascular endothelial cells.

Among the cellular constituents of heart muscle, both microvascular endothelial cells (CMEC) and cardiac myocytes exhibit a marked induction of the cytokine-inducible nitric oxide synthase (iNOS or NOS2) in response to soluble inflammatory mediators in vitro and in vivo in experimental animal models that mimic systemic sepsis or regional or global myocardial inflammation (1-8). Within the heart, for example, high levels of NO produced by cardiac myocytes or by CMEC following induction of NOS2 causes impaired myocyte contractile function that may contribute to the heart failure characteristic of the systemic inflammatory response syndrome or advanced cardiac allograft rejection (8-10). Unless the expression and activity of NOS2 are spatially and temporally regulated, NO and other highly reactive nitrogen oxide radicals can induce nonspecific cellular toxicity that may contribute to the death of the organism (11-14).

The regulation of NOS2 activity in most tissues is primarily at the transcriptional level, although post-transcriptional and post-translational regulatory mechanisms have been described (12, 14). In addition to interrupting selected components of the immune response that trigger NOS2 induction, glucocorticoids have been shown to suppress NOS2 activity. In ventricular myocytes and CMEC exposed to interleukin-1β (IL-1β) and interferon-γ (IFN-γ), for example, pretreatment with dexamethasone decreases NOS2 mRNA and protein abundance (4, 5). The mechanism(s) by which glucocorticoids suppress NOS2 induction in the presence of cytokines is unclear.

Other agents have also been shown to regulate the extent of induction of NOS2 by inflammatory mediators. Among these is osteopontin, a relatively ubiquitous extracellular matrix phosphoprotein that contains an RGD integrin-binding motif. Its function appears to be determined by the specific tissue and cell type from which it is secreted (15, 16). Hwang et al. (17) have recently shown that exogenous recombinant human osteopontin decreased both NOS2 mRNA abundance and enzyme activity in primary cultures of renal proximal tubular epithelial cells that had been exposed to lipopolysaccharide (LPS) endotoxin and IFN-γ. It was unclear, however, what regulated endogenous production and secretion of osteopontin in these cells.

Within the heart, it is not known which cell types express osteopontin nor what role, if any, osteopontin could have in the regulation of NOS2 expression in cardiac cells. In this report, we demonstrate that CMEC and ventricular myocytes constitutively express osteopontin mRNA in vivo and in primary culture, and that dexamethasone markedly increases osteopontin mRNA and secretion by both cell types. The data suggest that the suppression of NOS2 by glucocorticoids in both ventricular myocytes and in CMEC could be mediated in part by this multifunctional extracellular matrix phosphoprotein.

EXPERIMENTAL PROCEDURES

Cell Isolation and Culture

Cathal-sodiumventricular myocytes were isolated from hearts of adult male Sprague-Dawley rats (175-200 g) as described previously (13). Cells were plated in Dulbecco's modified essential medium (DMEM, Sigma), supplemented with albumin (2 mg/ml), L-carnitine (2

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**Immunoblot Analyses**

Detection of Osteopontin—Freshly isolated myocytes or confluent serum-starved CMEC cells were treated in 5 ml of medium with 3 μM dexamethasone in serum-free DMEM. Conditioned media were collected and adjusted with 0.2 ml phenylmethylsulfonyl fluoride. Media were centrifuged at 3,000 × g to remove cells and cellular debris and concentrated using Centricon 10 filters (Amicon, Inc.). Twelve μg of protein from each sample were resolved by 4–20% gradient SDS-PAGE (Bio-Rad). Proteins from the gel were electrophoretically blotted to 0.2 μm nitrocellulose membranes (Schleicher & Schuell). The membranes were stained with Ponceau S to confirm equal loading of the membranes before destaining. The membrane was incubated overnight in the blocking buffer (25 mM Tris (pH 7.5), 137 mM NaCl, 2.7 mM KCl, 0.2% Tween-20) containing 5% nonfat dry milk. The membrane was then incubated with monochonal anti-osteopontin antibody (Developmental Studies Hybridoma Bank, Iowa City, IA) diluted (1:20) in TBST containing 1% bovineserum albumin. Following washings with TBST, the membrane was incubated with a 1:10,000 diluted (1:20) of peroxidase-conjugated mouse anti-goat IgG. The immune complexes were detected using a chemiluminescence kit (NEN DuPont).

Detection of NOS2—Freshly isolated myocytes or confluent serum-starved CMEC cells were treated with cytokines (IL-1β and IFN-γ) for 16 h, with or without a 1-h preincubation in medium containing 20 mM synthetic osteopontin peptide (OPP). Cells were washed with cold phosphate-buffered saline and lysed with an immunoprecipitation buffer (1% Triton X-100, 150 mM NaCl, 10 mM Tris (pH 7.4), 1 mM EDTA, 1 mM EGTA, 0.2 mM sodium vanadate, 0.2 mM PMSF, 0.5 mM sodium orthovanadate, and P-40). Sixty and 90 μg of proteins obtained from CMEC, heart tissue, and myocytes, respectively, were resolved by 7.5% SDS-PAGE (Bio-Rad) and analyzed by immunoblot using an anti-NOS2 monoclonal antibody (Transduction Laboratories) as described above.

**RNA Isolation and Northern Analysis**

Total RNA was extracted from treated or untreated cardiac myocytes and CMEC by the method of Chomczynski and Sacchi (22). RNA was size-fractionated on 1.0% agarose gels containing 2.2M formaldehyde and transferred to nylon membranes (Gene Screen Plus; NEN DuPont) by using a transblot apparatus (Bio-Rad) in 10 × SSC (SSC is 0.15 M NaCl and 0.015 M citrate). Blots were hybridized overnight to radiolabeled probes in the presence of 1% SDS, 10% dextran sulfate, 1 mM NaCl, and 50% formamide at 42 °C. Full-length rat osteopontin (23) and partial-length type 2 NOS (5) cDNAs were labeled using a random prime labeling kit (Boehringer Mannheim). After hybridization, membranes were washed twice with 2 × SSC for 5 min each at room temperature, twice with 2 × SSC containing 1% SDS (for osteopontin) or 0.1% SDS (for NOS2) at 60 °C for 30 min, and twice with 0.05 × SSC at room temperature for 15 min prior to autoradiography. The same blots were then hybridized with an 18S oligonucleotide (30 mer) end-labeled by T4 polynucleotide kinase to normalize for loading differences (24). Differences in mRNA signal intensity were calculated using a 2202 Ultrascan densitometer (Pharmacia Biotech Inc.). Polyadenylated RNA was isolated from intact adult rat ventricular muscle using a kit (Invitrogen) exactly as described by the manufacturer. Five μg of poly(A)⁺ mRNA were analyzed by Northern blotting using the rat osteopontin cDNA probe as described above.

**Peptides**

Osteopontin Expression and Secretion by Cardiac Myocytes

A full-length rat osteopontin cDNA, isolated and characterized as described previously (23), was subcloned into the NotI site of the mammalian expression vector pCDNA3 (Invitrogen). The orientation of resulting clones was checked by restriction analysis and the plasmid containing the cDNA insert in antisense orientation was used to transfect primary cultures of microvascular endothelial cells that had attained approximately 50% confluency (i.e., 4–5 days after isolation and plating in DMEM + 20% fetal calf serum). The cells were washed twice with serum-free OPTI-MEM medium (without antibiotics; Life Technologies, Inc.), then transfected with 60 μg of Lipofectin and 40 μg of the antisense osteopontin cDNA in 3 ml of serum-free OPTI-MEM medium, according to the vendor’s instructions (Life Technologies, Inc.). Control cells were mock-transfected with Lipofectin alone in OPTI-MEM medium using the same protocol. The cells were incubated for 6 h before the medium was supplemented with DMEM and 20% fetal calf serum. After 48 h of transfection, CMEC were serum-starved for 3 h and then treated with cytokines for a further 16 h before analysis of osteopontin and NOS2 mRNA abundance.

**RESULTS**

Osteopontin Expression and Secretion by Cardiac Myocytes and Microvascular Endothelial Cells in Vitro and in Vivo: Regulation by Dexamethasone—Although several groups have reported data from tissue screens for osteopontin mRNA, only Giachelli et al. (18) have reported detecting osteopontin in normal rat myocardium, but at a level 50–60-fold lower than in normal rat aorta or carotid artery. To determine whether we could confirm this observation, poly(A)⁺ mRNA was isolated from ventricular muscle of two rat hearts. As shown in Fig. 1A, osteopontin mRNA could be detected readily by Northern blot in both samples. Osteopontin mRNA abundance also was examined in fresh primary isolates of adult rat ventricular myocytes and confluent primary cultures of CMEC by Northern blot. As shown in Fig. 1B, in the absence of inflammatory cytokines or dexamethasone, a 1.5-kilobase band consistent...
with osteopontin transcripts was readily detectable in RNA from CMEC, but only a faint band was observed in Northern blots of RNA from ventricular myocytes after much longer exposures of the autoradiogram.

The regulation of osteopontin expression is tissue-specific and complex in those cell types in which it has been examined (15, 16). In osteoblasts, agents that induce bone resorption including inflammatory mediators such as TNF-\(\alpha\), IL-1\(\beta\) and LPS, also induce osteopontin expression and secretion (26). To determine whether osteopontin expression could be enhanced in cardiac myocytes and CMEC, respectively, cells were exposed to a combination of cytokines (rhlIL-1\(\beta\), 4 ng/ml + rmIFN-\(\gamma\), 500 U/ml, lane 2) or dexamethasone alone (3 \(\mu\)M) (lane 3) for 16 h. The cells in lane 4 were pretreated with dexamethasone for 1 h, and then with the combination of cytokines for 16 h. Total RNA was extracted, and 15 \(\mu\)g of RNA were analyzed by Northern blot using \(^{32}\)P-labeled rat osteopontin cDNA as a probe. The filters were then hybridized to an 18 S probe to normalize for loading differences. The data are expressed as normalized osteopontin mRNA levels as a percent of control levels in the absence of dexamethasone or cytokines.

Osteopontin mRNA was detected in both myocyte and non-myocyte fractions from normal hearts. This signal could be increased severalfold at 16 h following injection of dexamethasone or of LPS, or with a combination of these agents (Fig. 3). A detectable increase above base-line osteopontin mRNA levels could be detected within 8 h of injection of LPS, with or without dexamethasone (data not shown).

To verify that these changes in mRNA abundance were paralleled by similar directional changes in osteopontin synthesis and secretion, the media conditioned by control or dexamethasone-treated cells were concentrated and analyzed by Western blot using a monoclonal anti-osteopontin antibody. As shown in Fig. 4A, a band corresponding to osteopontin (approximately 62 kDa) was detected in medium conditioned by CMEC. The intensity of this band was increased significantly in conditioned medium from dexamethasone-treated CMEC. In myocyte-conditioned medium, a slightly higher molecular mass band (approximately 69 kDa) was detected by the same antibody by Western blot. This band is within the range of sizes that has
been reported for this glycosylated phosphoprotein (i.e. 44–75 kDa) (16). As in CMEC, dexamethasone increased by approximately 3-fold the intensity of this 69-kDa band in proteins from ventricular myocytes (Fig. 4B). Similar data were obtained by immunoprecipitation of conditioned media from both cell types following metabolic labeling with [35S]methionine, using a polyclonal anti-peptide osteopontin antiserum prepared as described previously (23), followed by SDS-PAGE (data not shown).

Concentration Dependence and Time Course of Dexamethasone-induced Osteopontin Expression—Earlier studies (27) reporting suppression of osteopontin gene expression by dexamethasone in a rat osteoblast cell line (ROS 17/2.8) used lower dexamethasone concentrations (in the range of 30–100 nM) than were used to generate the data in Fig. 2 (i.e. 3 μM). To determine whether CMEC and cardiac myocytes also exhibited evidence of decreased osteopontin mRNA abundance at lower concentrations of dexamethasone, concentration-effect curves were performed that bracketed the concentrations used in these earlier studies and those used to generate the data in Fig. 2 (i.e. 30 nM to 15 μM). As shown in Fig. 5A, there was an increase in osteopontin mRNA levels above those observed in CMEC treated with vehicle alone at 30 nM, and this response increased with higher dexamethasone concentrations. An osteopontin mRNA hybridization signal was easily detected in adult cardiac myocytes at 30 nM and was maximal at 100 nM (Fig. 5B). At 3 μM dexamethasone, an increase in osteopontin transcript levels was apparent in microvascular endothelial cells within 1 h and continued to increase throughout the 16-h incubation period of the experiment (Fig. 6A). After addition of 3 μM dexamethasone, no increase in osteopontin transcript was detected by Northern blot until a 5-h time point in freshly isolated adult ventricular myocytes, and levels continued to increase through 16 h (Fig. 6B).

Dexamethasone and Osteopontin mRNA Stability—To determine the effect of dexamethasone on osteopontin mRNA stability, confluent serum-starved CMEC were treated with actinomycin D (10 μg/ml; Sigma) after 16 h of dexamethasone pretreatment. The results in Fig. 7 show that the half-life of osteopontin mRNA in control CMEC is approximately 11 h and is similar to that reported for ROS 17/2.8 cells by Noda et al. (28). There was a trend toward a slightly longer half-life for osteopontin in dexamethasone-pretreated cells, although this was not statistically significant. Thus, the principal mechanism by which glucocorticoids regulate osteopontin mRNA abundance appears to be at the transcriptional level.

Regulation of Inducible NOS2 Expression by Osteopontin—
Both microvascular endothelial cells and ventricular myocytes express NOS2 in situ in intact rat hearts and in vitro following exposure to soluble inflammatory mediators. To test the hypothesis that osteopontin secretion by one or both cell types in vitro could modulate NOS2 mRNA abundance, we first determined whether interruption of matrix protein-integrin binding affected NOS2 transcript levels. As shown in Fig. 8A, confluent serum-starved CMEC pretreated for 1 h with the synthetic hexapeptide GRGDSP (10 nM), which acts as an inhibitor of matrix ligand-integrin binding (29), increased NOS2 mRNA abundance by 50% after a subsequent 16-h incubation in the presence of IL-1β and IFN-γ over that of cells incubated in the absence of the hexapeptide. No NOS2 mRNA could be detected under basal conditions (i.e. in the absence of cytokines) or in cells incubated in the presence of the GRGDSP peptide alone. Higher concentrations of this synthetic hexapeptide did not result in any further increase in NO synthase transcript levels (data not shown) and led to some cell detachment from the plate. Similarly, in adult ventricular myocytes, as shown in Fig. 8B, GRGDSP (10–50 nM) enhanced NOS2 transcript levels by about 20–60% over those observed in myocytes treated with cytokines alone. These cells tolerated higher concentrations of the peptide without observable cellular detachment.

These data suggested that some matrix protein attachments, possibly involving endogenous osteopontin, could suppress the extent of NOS2 expression in both cell types. To determine whether osteopontin did affect the extent of induction of this NO synthase isoform, microvascular endothelial cells and ventricular myocytes were treated with a 20-mer synthetic peptide analogue (OPP) based on the rat osteopontin sequence that spans the RGD integrin-binding motif. OPP has been shown to be functional for osteopontin signaling and mimics recombinant human osteopontin in renal tubular epithelial cells (17). As shown in Fig. 9, 20 nM OPP decreased NOS2 transcript levels by approximately 50% in cytokine-pretreated microvascular endothelial cells. In cytokine-treated primary isolates of adult ventricular myocytes, there was about a 40% decline in NOS2 mRNA abundance at 20 nM and 50 nM OPP. This decline in NOS2 transcript with OPP also was accompanied by a reduction in NOS2 protein by approximately 20 and 50% in CMEC and adult ventricular myocytes, respectively, as detected by immunoblot analysis using an anti-NOS2 monoclonal antibody (data not shown).

Transfection of Antisense Osteopontin cDNA and NOS2 Expression in CMEC—To determine whether inhibition of endogenous osteopontin expression in microvascular endothelial cells could affect NOS2 mRNA abundance in cytokine-treated microvascular endothelial cells, primary cultures were tran-
siently transfected with a rat osteopontin antisense cDNA construct using Lipofectin. The cells were treated with cytokines following 48 h of transfection. Lipofectin alone did not affect osteopontin or NOS2 mRNA levels in cytokine-treated CMEC. Transfection with an antisense osteopontin cDNA reduced osteopontin mRNA by approximately 50% as compared to osteopontin mRNA levels in cells treated with cytokines alone or with Lipofectin alone (Fig. 10). However, NOS2 mRNA abundance was enhanced more than 2-fold in plates transfected with the antisense osteopontin cDNA construct (Fig. 10).

**DISCUSSION**

Osteopontin, originally identified in its role of facilitating resorption of bone hydroxyapatite by osteoclasts, is now known to be synthesized in many different cell types including luminal epithelial cells in many organs and by smooth muscle in a
number of tissues including the vasculature (27, 30–33). Osteopontin mRNA has been inconsistently detected in normal rat heart (18, 27, 34). Murry et al. (35) have reported that osteopontin expression was markedly increased in a subset of infiltrating macrophages around and within zones of myocardial injury induced by a transdiaphragmatic freeze-thaw technique. Similarly, Williams et al. (36) have recently reported that osteopontin mRNA is increased markedly in hearts of hamsters with a heritable cardiomyopathy, which they attributed to infiltrating tissue macrophages.

Osteopontin may play an important role in several cardiovascular disease processes, including atherosclerosis, aortic valve calcification, as well as repair of myocardial injury as reviewed by Giachelli et al. (37). An increase in osteopontin levels was observed in rat carotid arteries following vascular injury induced by experimental balloon angioplasty (18). Indeed, this extracellular matrix phosphoprotein may play a more general role in the immune response than mediating chemotaxis of phagocytic cells; for example, the early T-cell activation gene 1 (Eta1) that is expressed following nonspecific activation of several lymphocyte subclasses has been identified as being osteopontin (38). Osteopontin also can stimulate lymphocyte immunoglobulin production, suggesting that it may function as a cytokine in some circumstances (16). Murry et al. (35) also demonstrated that osteopontin expression could be detected in other tissues and cell types following injury, including regenerating skeletal muscle cells. Within the heart, osteopontin mRNA and protein has been detected by in situ hybridization and immunohistochemistry only within macrophages in injured muscle, but not in the extracellular matrix or other cell types (35, 36). Since the only known functions of osteopontin are related to its role as an extracellular matrix phosphoprotein, Murry et al. (35) speculated that extracellular osteopontin protein levels and presumably levels in otherwise normal heart muscle were below the level of detection by the immunohistochemical techniques they employed.

Denhardt and Guo (16), in a recent review, have emphasized the cell type specificity of the regulation of osteopontin gene expression. Osteopontin is expressed constitutively in arterial vascular smooth muscle cells and osteoblasts and its expression is increased by peptide growth factors such as basic fibroblast growth factor and transforming growth factor-β and by phorbol esters (16) and decreased by glucocorticoids, at least in the osteoblast cell line ROS 17/2.8 (27). In contrast, we find that glucocorticoids increase osteopontin expression and protein secretion in ventricular myocytes and CMEC in primary culture. This differential responsiveness to glucocorticoids may be due to the fact that the osteopontin promoter is known to contain two glucocorticoid response elements, as well as two AP-1 sites (16, 39, 40). Glucocorticoids, by binding to steroid hormone receptors, can directly repress AP-1-mediated transcriptional activation (41).

In addition to increasing osteopontin mRNA levels and protein content in ventricular myocytes and CMEC, dexamethasone also decreases NOS2 mRNA abundance and activity in both cell types (4, 5). The mechanisms by which glucocorticoids regulate cytokine-induced gene expression are complex and differ among specific cell types (42). However, the temporal association between osteopontin expression and decreased NOS2 mRNA levels suggests that endogenous osteopontin in the extracellular matrix could regulate NOS2 activity in these cells. This hypothesis is supported by the observation that the synthetic 20-mer osteopontin peptide analogue (OPP) decreased NOS2 mRNA and protein levels in both cell types and that transfection of CMEC with an antisense osteopontin cDNA decreased endogenous osteopontin mRNA levels while increasing NOS2 mRNA abundance in response to IL-1β and IFN-γ. The specific intracellular signaling pathways initiated by osteopontin binding to αvβ3 (or other) integrins that result in decreased NOS2 mRNA abundance are not known. Integrin recruited and autophosphorylated focal adhesion kinase, which can subsequently activate either ras or protein kinase C-dependent pathways, has been shown to synergistically enhance some cellular responses to cytokines (43). It is possible that

![Diagram](http://www.jbc.org/)

**Fig. 10. Transfection of CMEC with an antisense rat osteopontin cDNA construct increases NOS2 mRNA levels.** CMEC primary cultures that were approximately 50% confluent were transfed with an antisense osteopontin cDNA construct (AS OP cDNA) using Lipofectin for 6 h. As a control, cells were mock-transfected with Lipofectin alone. The media were supplemented with 10 ml of DMEM containing 20% serum for 48 h. The cells were then serum-starved for 3 h before exposure to rhIL-1β (4 ng/ml) and rmIFN-γ (500 units/ml) for a subsequent 16 h. Total RNA was isolated and analyzed by Northern blot using 32P-labeled osteopontin, NOS2, and 18 S probes. The normalized NOS2 and osteopontin hybridization signals are expressed relative to the NOS2 and osteopontin mRNA levels in CMEC treated with cytokines alone, the mean of which was set to 100% (*p < 0.01 compared to control NOS2 signal; **p < 0.02 compared to control osteopontin signal; mean ± S.E. of three experiments).
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Osteopontin acts to interrupt downstream signaling by other extracellular matrix proteins (such as fibronectin, with which osteopontin is known to interact) (25, 44, 45). Regardless of the specific mechanisms, it is likely that the increased expression, synthesis, and secretion of osteopontin induced by specific cytokines or glucocorticoids contributes to the spatial and temporal regulation of nitric oxide production by NOS2 in cardiac muscle.

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