Shear stress regulation of endothelial NOS in fetal pulmonary arterial endothelial cells involves PKC

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WEDGWOOD, Stephen, JANINE M. BEKKER, and STEPHEN M. BLACK. Shear stress regulation of endothelial NOS in fetal pulmonary arterial endothelial cells involves PKC. Am J Physiol Lung Cell Mol Physiol 281: L490–L498, 2001.—We have shown that increased pulmonary blood flow at birth increases the activity and expression of endothelial nitric oxide (NO) synthase (eNOS). However, the signal transduction pathway regulating this process is unclear. Because protein kinase C (PKC) has been shown to be activated in response to shear stress, we undertook a study to examine its role in mediating shear stress effects on eNOS. Initial experiments demonstrated that PKC activity increased in response to shear stress. NO production in response to shear stress was found to be biphasic, with an increase in NO release up to 1 h, a plateau phase until 4 h, and another increase between 4 and 8 h. PKC inhibition reduced the initial rise in NO release by 50% and the second increase by 70%. eNOS mRNA and protein levels were also increased in response to shear stress, whereas PKC inhibition prevented this increase. The stimulation of PKC activity with phorbol ester increased eNOS gene expression without increasing NO release. These results suggest that PKC may play different roles in shear stress-mediated release of NO and increased eNOS gene expression.

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Also, in the late-gestation fetal lamb, an infusion of the eNOS inhibitor l-NAME markedly attenuates the increase in pulmonary blood flow associated with ventilation at birth (1, 16). Taken together, these data strongly suggest that NO activity mediates, in part, the fall in pulmonary vascular resistance during the transitional pulmonary circulation and maintains the normal low postnatal pulmonary vascular resistance.

At least one important mechanism by which pulmonary vasodilation occurs relates to the increase in shear stress on the pulmonary vascular endothelium that is induced by the increase in pulmonary blood flow. Fluid shear stress is defined as the tractive force produced by moving a viscous fluid (blood) on a solid body (vessel wall), constraining its motion. The magnitude of shear stress increases with fluid viscosity (37). When endothelial cells (ECs) are subjected to shear stress, diverse responses are initiated, some of which occur within minutes and others that develop over many hours or days. Rapid changes occur in ionic conductance, adenylylate cyclase activity, inositol trisphosphate generation, and intracellular free calcium levels. If the shear stress is maintained, the rapid responses are then followed by much slower changes in gene expression and by structural reorganization of the cytoskeleton, producing changes in cell shape (36). NOS activity, NO production, and eNOS mRNA and protein expression are increased when ECs are exposed to increased shear stress (24, 27, 28, 38, 39, 42, 52, 53).

Previously, Black et al. (3) and Fineman et al. (16) have shown that the increased pulmonary blood flow occurring at birth leads to an increase in eNOS expression in the pulmonary vascular endothelium and that the NO released mediates, in part, both the immediate and the more gradual decreases in pulmonary vascular resistance after birth. However, the components of the second messenger system that transduce this signal are incompletely understood. Thus the purpose of this study was to begin to elucidate this signal transduction pathway to identify the shear stress-induced molecular events that regulate eNOS gene expression. We chose to investigate the role of the protein kinase C (PKC) system in transducing the shear stress signal because increasing evidence has shown that this pathway can be stimulated in ECs exposed to shear stress (26, 28, 39, 42, 52). Here, we demonstrate PKC-mediated changes in eNOS gene expression in response to laminar shear stress.

**MATERIALS AND METHODS**

**Cell culture techniques.** The heart and lungs were obtained from fetal (138–140 days gestation) lambs after death. These fetal lambs had not undergone previous surgery or study. The main and branching pulmonary arteries were removed and dissected free, and the adventitia was removed. The exterior of the vessel was rinsed with 70% ethanol. The vessel was then opened longitudinally, and the interior was rinsed with PBS to remove any blood. With a cell scraper, the endothelium was lightly scraped away, placed in medium DME-H16 (with 10% fetal bovine serum and antibiotics), and incubated at 37°C in 21% O₂-5% CO₂-balance N₂. After 5 days, islands of ECs were cloned to ensure purity. Basic fibroblast growth factor (1 ng/ml; a gift from Dr. Denis Gasparowicz, Chiron, Emeryville, CA) was added to the medium every other day. When confluent, the cells were passaged to maintain them in culture or frozen in liquid nitrogen. EC identity was confirmed by the typical cobblestone appearance, contact inhibition, specific uptake of acetylated low-density lipoprotein labeled with 1,1′-dioctadecyld-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiIC1, Molcular Probes, Eugene, OR), and positive staining for von Willebrand factor (DAKO, Carpinteria, CA). Ovine fetal pulmonary arterial ECs (OPFAECs) were studied between passages 3 and 10.

**Fluid shear stress.** A cone-plate viscometer similar to that described by Slogous et al. (46) was designed and built such that it accepts 15-cm tissue culture plates. This apparatus consisted of a cone of shallow angle (α) rotating at angular velocity (ω) on top of a tissue culture plate containing medium of a known viscosity (μ) on which ECs were present as a monolayer. This allowed the monolayer to be subjected to a radially constant fluid shear stress (τ dyn/cm²) as calculated with the formula τ = μωα/8. A cone angle of 0.5° was used to achieve laminar flow rates representing levels of shear stress within physiological parameters. Typical physiological shear stress in the major human arteries is in the range of 5–20 dyn/cm² (31), with localized increases to 30–100 dyn/cm² (12). Thus we imparted a shear stress of 20 dyn/cm² to mimic the upper limit of the physiological range. Also, because a previous study (39) showed that pulsatile and laminar flow appear to induce similar effects on NO production, laminar shear stress was employed.

**Generation of ovine eNOS cDNA and antiserum.** Oligonucleotides were synthesized (with bovine eNOS as a template) to allow amplification of this region within the ovine eNOS sequence. The sequences of the oligonucleotides were 5′-CCTCAGAGGGGGCCCCAGTTCTCCCTCCG-3′ for oligonucleotide 1 and 5′-CACGTCGAAGCGCGTTCGCGGGT-3′ for oligonucleotide 2. The region amplified corresponds to amino acids 62–288 of the heme-binding domain of the eNOS protein (48). Total RNA prepared from ovine fetal lung was used in RT-PCRs (kit from PerkinElmer, Foster City, CA). The cDNA fragment generated (681 bp) was then cloned directly into the pCR II vector (Invitrogen, San Diego, CA), sequenced (Sequenase kit from USB, Cleveland, OH), and used in RT-PCRs (kit from PerkinElmer, Foster City, CA). The sequence in this region for ovine eNOS is 96.6% identical to bovine eNOS.

**RNA isolation and analysis.** OPFAECs were grown on 15-cm plates and lysed with 4 M guanidinium thiocyanate. Total RNA was isolated by acid-phenol extraction (8). Because of its relatively low abundance, eNOS expression was evaluated by RNase protection assay according to our standard procedures (3). Briefly, single-strand antisense cRNA probes were synthesized, and then 50 μg of total RNA were hybridized overnight to 500,000 counts/min (cpm) of probe in 80% formamide-40 mM PIPES-0.4 M NaCl-1 mM EDTA at 42°C. This was followed by digestion with 5–10 U of RNase A and 25 U of RNase T1, phenol-CHCl₃ extraction, and ethanol precipitation. Protected fragments were then separated by electrophoresis on a DNA-sequencing gel and exposed to film at −70°C. Also included was a 170-bp cDNA fragment of ovine 18S to control for RNA input and recovery (3, 4).

**Protein preparation for Western blotting.** Cells were harvested in ice-cold PBS, centrifuged, resuspended in PBS, and sonicated before protein quantitation. One hundred micrograms of each protein extract were run on reducing SDS-
polyacrylamide gels. The gels were electrophoretically trans-
ferred to Hybond-polyvinylidene difluoride membranes,
blocked in PBS-Tween (0.1%) containing 5% nonfat dry milk,
and incubated with an eNOS monoclonal antibody (1:2,500
dilution; Transduction Laboratories). After being washed to
remove excess primary antibody, the membranes were incu-
bated with a secondary goat anti-mouse IgG conjugated with
horseradish peroxidase. After being washed to remove excess
secondary antibody, the bands were visualized with chemi-
luminescence procedures (3, 4).

Measurement of released nitrate. In solution, NO reacts
with molecular oxygen to form nitrite and with oxyhemoglo-
bin and superoxide anion to form nitrate (NO$_x$). The nitrite
and NO$_x$ were reduced with vanadium(III) and hydrochloric
acid at 90°C. NO was then purged from the solution, result-
ing in a peak of NO. Therefore, this value represents total
NO, nitrite, and NO$_x$. This peak was then detected by chemi-
luminescence (NOA 280, Sievers Instruments, Boulder, CO).
The detection limit was 1 nM/ml of NO$_x$.

Inhibition and stimulation and analysis of PKC activity.
PKC activity was determined with a commercially available
kit (Amersham).

Statistical analysis. Quantitation of autoradiographic re-
sults was performed by scanning (Microtek E6, Microtek,
Compton, CA) the bands of interest into an image-editing
software program (Adobe Photoshop, Adobe Systems, Mt.
View, CA). Band intensities from the RNase protection as-
says and the Western blot analysis were analyzed densito-
metrically on a Macintosh computer (model 9500, Apple
Computer, Cupertino, CA) with the public domain NIH Im-
ge program (developed at the National Institutes of Health
and available on the Internet at http://rsb.info.nih.gov/nih-
image). In the RNase protection assays, to control for the
amount of input RNA and the recovery of protected frag-
ments, the mRNA signal of interest was normalized to the
corresponding 18S signal for each lane. Results from un-
sheared cells were assigned the value of 1 (relative mRNA of
interest). For Western blot analysis, to ensure equal protein
loading, duplicate polyacrylamide gels were run. One was
stained with Coomassie blue. Results from unsheared cells
were assigned the value of 1 (relative protein of interest). The
means ± SD were calculated for the relative mRNAs and
proteins for each condition and were compared with the
unpaired t-test, with the use of the GB-STAT software pro-
gram. P < 0.05 was considered statistically significant.

RESULTS

After isolating the relevant late-gestation OFPAECs, we exposed
them to a controlled level of shear stress with a cone-plate viscometer similar to that described
by Sdogous et al. (46). Initial experiments with 20
dyn/cm$^2$ on OFPAECs demonstrated that the cells
changed morphology from a cobblestone appearance to
a more elongated shape, a feature of ECs under shear-
ing conditions (Fig. 1). We next measured cellular PKC
activity in OFPAECs exposed to shear stress (20 dyn/
$\text{cm}^2$ for 8 h). PKC activity was significantly increased
(7.9-fold, P < 0.05; Fig. 2). However, PKC activity was
not increased when cells were exposed to shear stress

![Fig. 1. Late-gestation ovine fetal pulmonary arterial endothelial cell (OFPAEC) shape and alignment change in response to fluid shear stress. Representative phase-contrast micrographs of confluent late-gestation OFPAECs exposed to 20 dyn/cm$^2$ of fluid shear stress for 0, 4, 8, or 24 h are shown. Direction of flow is from right to left.](image1)

![Fig. 2. Protein kinase C (PKC) activity in late-gestation OFPAECs exposed to fluid shear stress. Confluent late-gestation OFPAECs were exposed to 4 h of radially constant fluid shear stress (20 dyn/cm$^2$). Cell extracts were prepared and compared with baseline (untreated) cells or cells exposed to shear stress in the presence of a PKC inhibitor (PKCi; 1 $\mu$M staurosporine). N.D., not determinable. Values are means ± SD from 4 individual experiments calculated from triplicate readings. *P < 0.05 vs. baseline. †P < 0.05 vs. shear stress with PKC inhibition.](image2)
(20 dyn/cm² for 8 h) in the presence of the PKC inhibitor staurosporine (1 μM).

Next, we isolated RNA and protein from OFPAECs exposed to 20 dyn/cm² for 0–8 h in the presence and absence of PKC inhibition. Confluent late-gestation OFPAECs were exposed for 2, 4, and 8 h to radially constant fluid shear stress (20 dyn/cm²) in the presence and absence of the PKC inhibitor calphostin C (50 nM).

A: cRNA probe for ovine eNOS was hybridized overnight to 20 μg of total RNA prepared from OFPAECs. From baseline, eNOS mRNA expression increased after 2 h of shear stress and continued to increase further after 4 and 8 h. In the presence of PKC inhibition, this increase was abolished. No protected fragments were detected in the lanes where the probe was hybridized without RNA (probe alone (PA)) or in the presence of tRNA. eNOS is undigested probe. A cRNA probe for ovine 18S was also hybridized to serve as a control for RNA loading. Nos. at left, bp.

B: densitometric values for relative eNOS mRNA from 5 different experiments with different primary cultures. From baseline, shear stress increased relative eNOS mRNA by 2.3-, 3.2-, and 4.9-fold after 2, 4, and 8 h, respectively. In the presence of PKC inhibition with calphostin C, these values were 0.9-, 0.9-, and 0.56-fold after 2, 4, and 8 h, respectively. Values are means ± SD. *P < 0.05 vs. baseline.

C: protein extracts (25 μg) prepared from OFPAECs were separated on a 6% denaturing polyacrylamide gel, electrophoretically transferred to Hybond membranes, and analyzed with a specific antiserum raised against eNOS. Shear stress increased eNOS protein expression after an 8-h exposure. With PKC inhibition, this increase was abolished.

D: densitometric values for eNOS protein from 5 different experiments with different primary cultures. From baseline, shear stress increased relative eNOS protein by 1.12-, 1.62-, and 2.2-fold after 2, 4, and 8 h, respectively. In the presence of PKC inhibition, these values were, 0.97-, 0.99-, and 1.0-fold after 2, 4, and 8 h, respectively. Values are means ± SD. *P < 0.05 vs. baseline.

when PKC activity was attenuated with the specific PKC inhibitor calphostin C (50 nM), the shear stress-induced increase in eNOS gene expression was attenuated (Fig. 3). When other PKC inhibitors were used (staurosporine, H-7, and bisindolylmaleimide), similar results were obtained (data not shown), implicating PKC in the transduction of the shear stress signal transduction pathway, leading to an increase in eNOS gene expression.

Because NOS activity and hence NO production are increased when ECs are exposed to increased shear stress, we then determined if PKC inhibition would affect NO release from sheared OFPAECs. The results
obtained demonstrated that there was a biphasic rise in NO production in OFPAECs exposed to shear stress, with an initial rapid increase in NO release (0–1 h), a plateau phase (1–4 h), and then another increase in NO release (4–8 h; Fig. 4). This second increase in NO release corresponded to the time at which we could measure a significant increase in new eNOS protein (Fig. 3, C and D). Furthermore, NO release was reduced to a greater extent at 4–8 h compared with 0–1 h after PKC inhibition (Fig. 4B).

Finally, we determined whether the stimulation of cellular PKC activity altered either eNOS gene expression or eNOS activity in the absence of shear stress. When OFPAECs were exposed to the PKC activator phorbol 12-myristate 13-acetate (PMA; 300 nM) for 4 h, the expression of eNOS mRNA was shown to increase by 3.1-fold over that in cells treated with vehicle alone ($P < 0.05$; Fig. 5). However, there was no concomitant increase in the level of NO released from the PMA-treated cells compared with those exposed to vehicle alone (Fig. 6A). This lack of NO release from PMA alone did not appear to be a result of a PKC-mediated inhibition of the eNOS enzyme because OFPAECs pretreated with PMA before exposure to shear stress produced equivalent levels of NO to those exposed to shear alone (Fig. 6B). These results suggest that the role of PKC may be different in the signal transduction pathways that are activated by shear stress to stimulate NO production or eNOS gene expression.

**DISCUSSION**

In the fetus, pulmonary vascular resistance is high, and pulmonary blood flow is low. At birth, there is a rapid 8- to 10-fold increase in pulmonary blood flow related to a marked decrease in pulmonary vascular resistance. These rapid changes in pulmonary blood flow and resistance are then followed by more gradual changes that occur during the next several hours. There is increasing evidence that the changes in pulmonary vascular tone are mediated by NO, possibly in response to increased shear stress on the pulmonary vascular endothelium (17, 21, 40, 41, 47). In a number of clinical conditions, there is a failure of the pulmonary circulation to undergo the normal transition to postnatal life, resulting in persistent pulmonary hypertension of the newborn (2, 4, 35). A better understanding of the mechanisms responsible for the pulmonary vascular changes at birth may lead to new prevention of and improved treatment strategies for persistent pulmonary hypertension of the newborn. However, the signaling mechanisms involved in mediating these changes remain unclear.

To allow a molecular analysis of the effects of birth on the fetal pulmonary endothelium, we first isolated and cultured the relevant cells exposed to increased shear stress at birth. These cells were late-gestation OFPAECs (135–140 days gestation). To mimic the effects of increased blood flow, we used a cone-plate viscometer that allowed us to expose the OFPAEC monolayer to a controlled level of shear stress. The laminar shear stress produced by this technique has previously been shown (39) to induce NO production and to upregulate the level of eNOS mRNA in human umbilical vein ECs (HUVECs). Our results indicate a
biphasic production of NO by OFPAECs in response to shear stress. The initial increase in NO production between 0 and 1 h may have been due to the activation of preformed eNOS enzyme. In contrast, the second phase of NO production, occurring after 6–8 h of shear stress, corresponded with an increase in levels of eNOS mRNA and protein and may have been stimulated by factors that regulate eNOS gene expression. The eNOS gene 5'-flanking region contains a cis-acting regulatory sequence identical to the previously identified shear stress-responsive element (43), raising the possibility that shear stress-induced factors may control eNOS gene transcription via this potential shear stress-responsive element.

Shear stress has been shown to regulate the expression of a variety of genes that encode proteins affecting transcription, cytoskeletal structure, apoptosis, release of vasoactive substances, smooth muscle proliferation, and EC growth arrest (10, 31). Recently, the effects of shear stress on the endothelin system were studied in HUVECs (34). This work demonstrated a downregulation of endothelin gene expression by shear stress, which was prevented by the eNOS inhibitor Nω-nitro-L-arginine methyl ester. It seems likely that the NO and endothelin systems regulate each other via an autocrine feedback loop (32). These data identify a role for shear stress in the control of gene expression within the NO and endothelin cascades and further illustrate the complex interactions between them. However, the precise mechanisms involved remain to be determined.

Increasing evidence has shown that the PKC system is stimulated in ECs exposed to shear stress (26, 28, 39, 42, 52). We thus undertook a study to determine if shear stress-stimulated PKC activity can transduce the signal to activate eNOS. The results obtained clearly demonstrated that the PKC system was both necessary and sufficient to induce eNOS gene expression. However, although PKC was found to play an important part in the stimulation of NO release, both initially and after 6–8 h of exposure to shear stress, PMA-induced activation of PKC alone was insufficient to activate the eNOS enzyme. PKC activation is thought to occur in response to the synergistic action of diacylglycerol and calcium, both of which can be generated by signal-induced hydrolysis of membrane phospholipids. Thus intracellular calcium might be increased on exposure of OFPAECs to PMA. Because eNOS is calcium dependent, it might have been expected that NO production would be stimulated. However, we did not obtain this result. Because a previous study (18) suggested that PKC-mediated phosphorylation may inhibit eNOS activity, it was possible that PMA was inhibiting eNOS, reducing its ability to pro-

Fig. 5. eNOS mRNA expression in the presence and absence of the PKC activator phorbol 12-myristate 13-acetate (PMA). Confluent late-gestation OFPAECs were exposed to PMA (300 nM) or vehicle alone for 4 h. A: cRNA probe for ovine eNOS was hybridized overnight to 20 μg of total RNA prepared from OFPAECs. From baseline, eNOS mRNA expression increased in the presence of PMA. No protected fragments were detected in the lanes where the probe was hybridized without RNA (PA) or in the presence of tRNA. eNOS is undigested probe. A cRNA probe for ovine 18S was also hybridized to serve as a control for RNA loading. UTD, untreated; nos. at left, bp. B: densitometric values for relative eNOS mRNA from 5 different experiments with different primary cultures. From baseline, shear stress increased relative eNOS mRNA 3.2-fold after 4 h. Values are means ± SD. *P < 0.05 vs. baseline.

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duce NO. However, this was not the case because OFPAECs produced similar amounts of NO whether cells were sheared in the presence or absence of PMA. Our results suggest that PKC may have two distinct roles in the transduction of the shear stress signal, leading to NO production in OFPAECs. The contribution by PKC to the initial response that results in increased eNOS enzyme activity may be different from its contribution in the longer-term response that results in increased eNOS gene expression.

PKC can be measured in both cytosolic and particulate fractions, and different tissues appear to have characteristic patterns of expression of PKC isozymes, leading to the hypothesis that each isozyme has a different biological role. Our results have identified PKC as one signal transduction molecule involved in regulating eNOS activity and gene expression by fluid shear stress. In fact, shear stress has been shown to elevate PKC-β immunostaining in HUVECs, with increased translocation to the cell membrane (19). Furthermore, it has been demonstrated that PKC-ε is activated by shear stress and plays a role in transducing the shear stress signal, resulting in the activation of extracellular signal-regulated kinase 1/2 (51), whereas PKC-dependent activation of extracellular signal-regulated kinase 1/2 has also been shown in bovine aortic ECs in response to cyclic strain (20). A previous study (30) found that PMA incubation produced the translocation of PKC-α and PKC-ε from the cytosol to the cell membrane in HUVECs, indicating activation of these isoforms. When these cells were transfected with a 3.5-kb fragment of the human NOS III promoter driving a luciferase reporter gene, PMA stimulated promoter activity up to 2.5-fold (30). In addition, PMA enhanced basal and bradykinin-stimulated NO production and stimulated eNOS mRNA expression in a concentration- and time-dependent manner (30). Specific PKC inhibitors also prevented the upregulation of NOS III mRNA produced by PMA. These data suggest that stimulation of PKC is involved in the signaling pathway activating the human eNOS gene promoter in human endothelium.

The expression of PKC isoforms has been studied in bovine aortic ECs with immunohistochemical and Western blotting techniques (44, 51) and in cerebral ECs with RT-PCR. The results obtained are interesting because each cell type appears to express a different spectrum of PKC isozymes: bovine aortic ECs have been shown to express the PKC-α, -δ, -ε, and -ζ isoforms, whereas cerebral ECs expressed the α, β, δ, ε, and η isoforms. These results suggest that ECs can express a different complement of PKC isoforms in different areas of the circulation. The PKC expression pattern of the ECs of the pulmonary vasculature has not been determined and may thus be important in identifying the mechanisms that induce eNOS expression in response to increased fluid shear stress.

In summary, the involvement of PKC in shear stress-induced NO production appears to occur at the level of eNOS enzyme activity and eNOS gene expression. It seems likely that a subset of PKC isoforms provides

Fig. 6. NOx release from late-gestation OFPAECs exposed to the PKC activator PMA in the presence and absence of fluid shear stress. A: confluent late-gestation OFPAECs were exposed to PMA (300 nM) or vehicle alone for 4 h. Samples of medium (5 μl) were removed and analyzed for NOx content with a NOA 280 analyzer to detect the chemiluminescence peak. Values are means ± SD from 3 individual experiments calculated from triplicate readings. There was no increase in the release of NOx in the presence of PMA. B: confluent late-gestation OFPAECs were exposed to PMA or vehicle alone for 1 h and then exposed to radially constant fluid shear stress (20 dyn/cm²) for 60 min. Samples of medium (5 μl) were removed and analyzed for NOx content with the use of an NOA 280 analyzer to detect the chemiluminescence peak. Values are means ± SD from 6 individual experiments after normalization to NOx released from vehicle-treated cells exposed to shear stress. There was no significant difference in the release of NOx in the presence of PMA in response to shear stress.
distinct functions, giving rise to several different signaling pathways. As yet, these isoforms and their specific roles remain to be elucidated. In addition, the PKC-independent pathways that also result in NO production in response to shear stress have yet to be determined. Further studies are required to identify the mechanisms involved.

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