The 67-kDa Laminin-binding Protein Is Involved in Shear Stress-dependent Endothelial Nitric-oxide Synthase Expression*

(Received for publication, September 28, 1998, and in revised form, February 25, 1999)

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It has been suggested that the mechanical forces acting on endothelial cells may be sensed in part by cell-matrix connections. We therefore studied the role of different matrix proteins, in particular laminin I, on a shear stress-dependent endothelial response, namely nitric-oxide synthase (eNOS) expression. Primary porcine aortic endothelial cells were seeded onto glass plates either noncoated (NC cells) or precoated with fibronectin (FN cells), laminin (LN cells), or collagen I (CN cells). Western blots were used to detect differences in the final matrix composition of these cells. A shear stress of 16 dyn/cm² was applied for 6 h. Only LN cells showed detectable amounts of laminin I in their underlying matrix when they reached confluence. They reacted with a 2-fold increase of eNOS expression (n = 16, p < 0.001) to the exposure of shear stress, which went along with enhanced eNOS protein and NO release. In contrast, neither FN cells (n = 9) nor NC cells (n = 13) showed a significant increase of eNOS expression under shear stress. The increase in CN cells was borderline (1.4-fold; n = 9, p < 0.05) and was not associated with an increase of eNOS protein. The shear-induced increase in eNOS expression of LN cells was abolished by the peptide YIGSR, which blocks the cellular binding to laminin I via a 67-kDa laminin-binding protein, whereas a control peptide (YIGSK) had no effect. The induction of eNOS expression by shear stress is stimulated by an interaction of endothelial cells with laminin which is, at least in part, mediated by a 67-kDa laminin-binding protein.

Shear stress, elicited by blood flow, represents an important signal for endothelial cells to synthesize vasoactive autacoids. In addition to an acute increased autacoid production there are also chronic effects of shear stress. One such effect is the differential expression of some proteins in endothelial cells (1, 2). It has been shown by several groups that, in particular, the expression of the endothelial nitric-oxide synthase (eNOS) is increased when endothelial cells are exposed to laminar shear stress for several hours (3–10). To date, our knowledge of the exact signal transduction cascade from the stimulus (shear stress) to the effect (gene expression) still remains incomplete. In particular, little is known about the role of the extracellular matrix in this signaling process, although several lines of evidence support a role for the cell-matrix interaction in shear stress-induced cell activation (11–14).

Several authors have reported an alignment of endothelial cells in the direction of the shear force applied to cultured cells and blood vessels (13–18). Such a reorientation must involve the formation of new cell-matrix contacts. Indeed, focal adhesion points change dynamically in endothelial cells under shear stress (19). These focal adhesion points represent not only the connection between matrix and cell membrane but also are colocated in a number of regulated kinase activities in these sites, which may be involved in the translation and transduction of extracellular signals across the cell membrane (20). If such focal adhesion complexes are active as a link between the shear stress and subsequent gene expression, the protein composition of the extracellular matrix should play a very important role.

Among these matrix proteins, laminins represent the major glycoprotein family in all basement membranes and as such are of particular interest. In addition, the cellular laminin secretion under shear stress appears to be up-regulated, whereas fibronectin seems to be down-regulated (21–23). Previous studies have shown that members of the laminin family specifically promote cell growth, differentiation, and migration (24). Data support that part of these effects are dependent on a so-called “laminin-binding protein” (LBP) of 67 kDa in size (25). This 67-kDa LBP was identified to bind highly selectively to a certain sequence of the laminin β1 chain, namely YIGSR, and to promote cell attachment as well as migration (26, 27).

In this study laminin I was examined for its potential role in the shear stress-mediated expression of the eNOS. Special attention was given to the laminin-cell interaction via the non-integrin 67-kDa LBP and its involvement in the signal transduction of shear stress-mediated eNOS expression.

There is ample literature about the 67-kDa LBP involvement at the pathophysiologic level, such as tumor invasion; however, there is little mention of a physiologic role for the LBP. It was our reasoning that the function of the 67-kDa LBP in the above mentioned signal cascade downstream of the shear stress could represent a major physiological task of this LBP in the vascular system.

EXPERIMENTAL PROCEDURES

Cell Culture—From fresh porcine aorta obtained from the local slaughterhouse, endothelial cells were isolated following standard procedures (28). Briefly, the fat and connective tissue were trimmed from the aorta with sterile scalpels, and the vessel was cut longitudinally. After washing with sterile phosphate-buffered saline (PBS), vessels were put into a frame with endothelial side facing up. At this point they were incubated with dispase (Boehringer Mannheim; 2.4 units/ml PBS) for 20 min at 37 °C in a humidified incubator. Endothelial cells were dislodged with sterile medium (Dulbecco’s modified Eagle’s and Ham’s F-12 media, 1:1), collected, and thereafter seeded onto standard plastic culture dishes in Dulbecco’s modified Eagle’s and Ham’s F-12 media (1:1) containing penicillin/streptomycin, glutamine (200 mM), and 20% fetal calf serum (Biomol). The cell culture medium was replaced every
other day. Only cells from passage 1 were used for shear stress experiments, and the corresponding control cells were obtained from identical pools.

Coating of Glass Plates, Application of Shear Stress and Inhibitory Peptides—Plastic culture dishes were not suitable for the shear stress experiments, nor were they not photoreactive. Therefore, coverslips were used for a high degree of variability as to the bottom thickness. Therefore glass plates were used to create a monolayer of endothelial cells for the application of shear stress. Before inoculation with the cells, the plates were coated according to standard procedures. Briefly, clean glass plates were each incubated with 20 g/ml laminin I (from Engelbreth-Holm-Swarm tumor purchased from Sigma), laminin fragments (10 g/ml each), or a generous gift from Lydia Sorokin, Erlangen), collagen I (from Boehringer), fibronectin (human sera purchased from Boehringer), or they were used without any coating. The endothelial cells were allowed to adhere and were cultivated up to confluence (approximately 3 days). The glass plates were then placed into a rotating cone shear apparatus. For a glass plate with a diameter of 10 cm, an angle of 1° of the cone guaranteed a homogeneous laminar shear stress. The rotating speed was adjusted in a way so that the shear stress generated was 16 dyn/cm². Incubations were performed in complete culturing medium with reduced serum content (1% fetal calf serum for 6 h).

Attachment Experiments—Attachment experiments were done following the paper of Iwamoto et al. (26). At the time of seeding, 150 g/ml of the YIGSR peptide or YIGSK for control was added. After various intervals, cellular attachment was recorded microscopically by counting cells in 10 randomly distributed optical fields.

Acute effects of YIGSR on attachment, in the presence of shear stress, were tested as follows. Endothelial cells were allowed to adhere to laminin I for 16 h, and the peptide YIGSR (150 g/ml) was added just before the shear stress. All attachment experiments were done in triplicate.

To test the effects on shear stress-mediated signal transduction via the 67-kDa LBP, the inhibitor peptide YIGSR against LBP as well as the control peptide YIGSK were added at a much lower concentration (35 g/ml each), 3 days after initial seeding and immediately before the onset of shear stress.

Northern Blots—After applying shear stress to the porcine aortic endothelial cells the cell medium was aspirated. To the cell layer 2 ml of Trizol reagent (Life Technologies, Inc.) for each 10-cm dish was added, and total RNA was isolated following the instruction of the company. 10–20 g of total RNA was denatured and loaded onto a formaldehyde/formamide hybridization gel. After completion of the electrophoresis, the RNA was transferred to nylon membranes (Nytran, Zymark) with UV light (320 nm, 0.7 J/cm²) for 3 h at 42 °C. A randomly labeled cDNA probe for eNOS (base pairs 3107 to 3405) was added with fresh buffer and incubated for 24 h at 42 °C. After washing twice for 15 min at room temperature in 2× SSC and 0.1% SDS, the filter was exposed to autoradiographic films. Finally, this ratio for the cells under shear stress was compared with its control under static incubation.

Semiquantitative Reverse Transcriptase-PCR—Alternatively to Northern blots, the eNOS mRNA expression was assessed by semiquantitative reverse transcriptase-PCR. The extraction of total RNA was the same as for Northern blots. The primers used were 5′-CTCTTCCAGGCTCCTGTTGAGCTT- (2116–2140) and 5′-ACGTGGGACGACGTCATAGTGCA-3′ (2948–2924) from porcine eNOS mRNA. Following the protocol of the manufacturer (Titan kit from Boehringer), all necessary components of the enzyme mix and the specific primers were pipetted together in a 2× setup. At this point the mix was divided into two halves, and the primers for eNOS and GAPDH were added. Loading discrepancies were thus reduced to a minimum. The number of cycles in which the PCR was still in the linear range was tested out in previous experiments, and the relative expression of eNOS mRNA was calculated according to the Northern blot experiments by normalization of eNOS bands to their corresponding GAPDH bands.

Analysis of Matrix Protein Composition—Even after 3–5 days, which the endothelial cells needed to reach confluence, there was a clear distinction with regard to the matrix composition of cells grown on laminin I, collagen I, fibronectin, or glass alone. Although the macroscopic inspection of the matrix indicated no differences (see Coomassie-stained gel in Fig. 1), no laminin I could be detected on collagen I, on fibronectin precoated or on pure glass plates, whereas on laminin I-coated plates a clear laminin I signal was obtained (see Fig. 1C). Fibronectin, however, was found in each matrix composition regardless of the previous coating procedure.

In contrast to the findings above, when plastic dishes were
used instead of glass plates, we always found laminin I in the matrix, regardless of the initial coating with fibronectin, collagen I, or laminin I (data not shown).

Expression Analysis of eNOS—Alterations in the expression level of the eNOS mRNA were analyzed with Northern blots as well with reverse transcriptase-PCR. Fig. 2A shows a typical Northern blot. The upper row represents the hybridization signal with the eNOS probe, whereas the lower row shows the one for GAPDH.

Note that for laminin I coating, the cells showed an apparent increase of eNOS mRNA. No obvious change in the eNOS expression was found in the case of coatings with collagen I and fibronectin as well as without any coating. The GAPDH expression showed no alterations caused by shear stress or coating procedure.

In Fig. 2B the results of different experiments are summarized. The relative expression of eNOS in endothelial cells after a 6-h shear stress (16 dyn/cm²) was not significantly altered in the case of coatings with collagen I and fibronectin as well as without any coating. The GAPDH expression showed no alterations caused by shear stress or coating procedure.

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growth factor. There were no differences between cells incubated with or without the peptide YIGSR, indicating that viability as well as proliferation was not altered by YIGSR (data not shown).

Inhibition of Shear Stress-induced eNOS Expression—Both semiquantitative reverse transcriptase-PCR and Northern analysis showed that the original 2-fold laminin-dependent increase of the eNOS expression after shear stress was abolished during incubation with the peptide YIGSR ($n = 5$) (see Fig. 7). In contrast, control experiments with the peptide YIGSK had no significant effect on the shear stress-dependent expression pattern (2.3-fold induction, $n = 6$).

These findings went along with experiments using the enzymatic laminin fragments E8 or P1. In contrast to P1, the E8 fragment does not contain the region where the 67-kDa laminin receptor binds to the molecule because it represents only the “stem” portion of the laminin cross-shaped trimer. Coating with the E8 fragment showed no induction after a 6-h shear stress (1.2-fold over control, $n = 2$), whereas coating with P1 yielded a higher response to the shear stress (3.5-fold increase of expression, $n = 3$).

Effects of Incubations with YIGSR on eNOS Protein after Shear Stress—To verify the results of the previous mRNA analysis we performed also Western blots against eNOS protein. Western blot experiments for the different coatings collagen I, fibronectin, and glass alone showed no induction of the eNOS protein after a 6-h shear stress (Fig. 3B shows representative bands of $n = 2$). However, as in Fig. 3A, endothelial cells grown on laminin I showed a remarkable increase of eNOS ($n = 4$). This protein induction was abolished completely by an incubation of the cells with the LBP-inhibitory peptide YIGSR during shear stress ($n = 3$) (see Fig. 8, A and B), whereas incubation with the control peptide YIGSK had no effect ($n = 3$).

**DISCUSSION**

These experiments demonstrate that the application of shear stress over several hours augments the expression of eNOS in porcine endothelial cells. The new finding in this study is that the augmentation of eNOS expression depends on the presence of laminin I in the extracellular matrix, suggesting a modulator role for it in the response of a vessel to mechanical forces. A 67-kDa LBP, which seems to be constitutively expressed in the endothelium, acts as a functional sensor at the basal side of the cell and is crucially involved in the translation of the physical force to gene expression.

The increase of eNOS expression upon shear stress is in accordance with earlier reports in cultured bovine aortic (3–6) and human umbilical vein endothelial cells (5, 8, 30). Similar results have been reported under in vivo conditions in lamb pulmonary arterial (9) and rat aortic (10) as well as in canine coronary endothelial cells (31).

Some groups observed an increase of eNOS expression in cells being cultured on non-laminin matrices (3, 30). This is in apparent contrast to our results because cultivating cells on fibronectin or pure glass abolished the shear stress sensitivity in terms of eNOS expression. This discrepancy may be explained by variations in the ability of the cells to produce endogenous laminin. Our observation that cells taken from the same pool produced high amounts of laminin I when grown on plastic (irrespective of the coating) but not when cultivated on glass plates, except when the glass plates were precoated by laminin, supports the explanation above. Indeed, our Western blot data suggest a laminin I-induced laminin production under these conditions.

Therefore, by cultivating endothelial cells on glass plates, a specific role of laminin I could be detected when compared with
other matrix proteins. This is not an effect of laminin I alone because the addition of laminin I to the superfusate did not change the eNOS expression in control cells kept under static conditions (data not shown). Only in combination with shear stress was there an effect (a 2-fold increase of eNOS expression). Interestingly, it has been reported that endothelial cells under chronic shear stress produce more laminin at the expense of fibronectin (21, 22). Additionally, laminin expression correlates with endothelial cell differentiation during angiogenesis (24, 32, 33). There is circumstantial evidence that eNOS expression also correlates with endothelial cell differentiation (34).

Binding of the cell to laminin I occurs not only via integrins, especially those consisting of a β1- and one of several α-chains but also by a 67-kDa LBP (25–27). LBP was constitutively expressed, regardless of the underlying matrix, which may reflect its potential significance in mediating effects of matrix proteins on cell signaling. The presence of a functioning LBP at the membrane level was assessed indirectly by testing the adherence of freshly dispersed cells to a laminin matrix. High

FIG. 3. Western blot for eNOS protein. Panel A, a representative Western blot is shown of total cell lysates from porcine aortic endothelial cells grown on laminin I-precoated plates either under static control conditions or under a 16-dyn/cm² shear stress for 6 h. Panel B, representative Western blots of total cell lysates from endothelial cells grown on fibronectin, pure glass, or collagen I, which were subjected to 16 dyn/cm² for 6 h. All experiments were quantified with a videodensitometer and normalized to the static control, which was set at 1. Panel C, laminin I coating (n = 4, p = 0.054); panel D, collagen I, fibronectin, and pure glass (n = 2 each).

FIG. 4. Nitrate production under shear stress. Porcine aortic endothelial cells were grown on a laminin matrix and exposed to a 16-dyn/cm² shear stress. After 6 h the experiments were stopped, and nitrate in the supernatant was measured with the cadmium reduction method and normalized to total cellular protein.

* p<0.05, n=11

FIG. 5. Western blots for the 67-kDa LBP. Panel A, porcine aortic endothelial cells were grown under static conditions either on pure glass or with a precoating of the culture plate with collagen I, fibronectin, laminin I. Panel B, porcine aortic endothelial cells grown on laminin I were subjected to shear stress of 16 dyn/cm² for 6 or 16 h. Equal amounts of cell lysates were separated on reducing SDS-polyacrylamide gel, blotted onto nitrocellulose, and probed with an antibody specific for 67-kDa LBP.
concentrations of the inhibitor of LBP binding, YIGSR (see below), reduced the adherence and led to an extensive detachment of freshly attached cells under shear stress. This may be the result of the lack of other attachment substrates. After 2–3 days in culture and using much less peptide, this shear-induced detachment was not observed, indicating that binding sites other than LBP are more important for permanent attachment and firm cell adherence. These hypothesis is supported by macroscopic inspection of the matrix protein compositions. The Coomassie-stained protein gels showed no obvious differences among the various pretreatments of the culture plates. However, when probed in Western blots there was a clear distinction between the laminin coating and collagen and fibronectin coating. This suggests that the role of LBP is not primarily in cell adhesion but perhaps in sensing external signals applied to the cell.

Even after several days in culture, the laminin I-dependent eNOS expression could still be inhibited by the addition of YIGSR, a peptide deduced from the LBP binding site (27) on the laminin I molecule. When applied in high concentrations, this peptide was shown to attenuate tumor invasion and vas-

FIG. 6. Attachment experiments of endothelial cells to laminin I. Panel A, simultaneous with the time point of seeding the porcine aortic endothelial cells onto a laminin I-coated plate the LBP inhibitory peptide YIGSR was added at a high concentration (150 μg/ml). The subsequent cellular attachment was recorded by counting the cells in 10 randomly distributed optical fields. Each experiment was done in triplicate. Panel B, shortly after first adhesion to laminin I (16 h) endothelial cells were subjected to a shear stress of 16 dyn/cm² for 6 h. The addition of a high dose of the LBP inhibitory peptide (150 μg/ml) resulted in an 80% loss of cells, whereas with no peptide addition less than 10% of the cells was dislodged. Each experiment was done in triplicate.

FIG. 7. Influence of the YIGSR peptide on shear stress-induced eNOS expression. RNA from porcine aortic endothelial cells grown on laminin was inoculated without any peptides, with the LBP-inhibitory peptide YIGSR, or with its control peptide YIGSK. After an exposure of a shear stress of 16 dyn/cm² for 6 h, total RNA was isolated, and 10–20 μg was separated on a formaldehyde/formamide agarose gel. Videodensitometric quantification of several Northern blots was analyzed as described. The densities of specific eNOS mRNA bands of all experiments were related to their corresponding GAPDH bands. The relative expression represents the eNOS mRNA of the shear stress experiments normalized to their static control, which was set at 1.

FIG. 8. Western blots for eNOS protein. Panel A, representative Western blot of total cell lysates from porcine aortic endothelial cells grown on laminin I-precoated plates either under static control (C) conditions or under 16-dyn/cm² shear stress with the LBP-inhibitory peptide YIGSR (+R) or with the control peptide YIGSK (+K) for 6 h. Panel B summarizes several experiments (n = 3 for peptides and n = 4 without peptides) that were analyzed densitometrically and normalized to their corresponding static control.
the expression of eNOS was increased. In fact, the increase in eNOS expression went along with the fragment E8 of laminin, which represents the large stem of the protein that does not contain the binding site for LBP. This very close association of the protein and translational level completely. This inhibition was highly specific because the nearly identical control peptide had no effect. The central role of LBP in mediating the signal transduction pathway by which shear stress elicits eNOS expression. This function suggests a physiological role for a protein that, up to this point, was considered important mainly for pathophysiological processes such as tumor invasion.

Acknowledgments—We gratefully acknowledge the scientific language editing by Sarah Neuhaus, and we thank B. Doerge for help in preparing the figures.

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