

Role of hyaluronic acid glycosaminoglycans in shear-induced endothelium-derived nitric oxide release

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Mochizuki, Seiichi, Hans Vink, Osamu Hiramatsu, Tatsuya Kajita, Fumiyuki Shigeto, Jos A. E. Spaan, and Fumihiko Kajiya. Role of hyaluronic acid glycosaminoglycans in shear-induced endothelium-derived nitric oxide release. *Am J Physiol Heart Circ Physiol* 285: H722–H726, 2003. First published May 1, 2003; 10.1152/ajpheart.00691.2002.— Endothelium-derived nitric oxide (NO) is synthesized in response to chemical and physical stimuli. Here, we investigated a possible role of the endothelial cell glycocalyx as a biomechanical sensor that triggers endothelial NO production by transmitting flow-related shear forces to the endothelial membrane. Isolated canine femoral arteries were perfused with a Krebs-Henseleit solution at a wide range of perfusion rates with and without pretreatment with hyaluronidase to degrade hyaluronic acid glycosaminoglycans within the glycocalyx layer. NO production rate was evaluated as the product of nitrite concentration in the perfusate and steady-state perfusion rate. The slope that correlates the linear relation between perfusion rate and NO production rate was taken as a measure for flow-induced NO production. Hyaluronidase treatment significantly decreased flow-induced NO production to $19 \pm 9\%$ of control (mean \pm SD; $P < 0.0001$ vs. control; $n = 11$), whereas it did not affect acetylcholine-induced NO production ($88 \pm 17\%$ of pretreatment level, $P =$ not significant; $n = 10$). We conclude that hyaluronic acid glycosaminoglycans within the glycocalyx play a pivotal role in detecting and amplifying the shear force of flowing blood that triggers endothelium-derived NO production in isolated canine femoral arteries.

glycocalyx; canine femoral artery; hyaluronidase; nitrite; shear stress

VASCULAR ENDOTHELIUM RELEASES numerous vasoactive factors, including nitric oxide (NO), prostaglandins, and endothelins, in response to various chemical (agonist) and physical (blood flow and pressure) stimuli. Recently, we demonstrated (12) a linear relation between perfusion rate (shear stress) and endothelial NO concentration in the vascular media of isolated, perfused canine femoral arteries. The flow-induced shear stress detection mechanisms of the endothelial cells have been studied extensively, including the cytoskel-

eton (4), phospholipid bilayer (6), and changes in cell membrane fluidity (2, 7).

The biological functions of the glycocalyx have been studied in various aspects including regulation of vascular permeability (1) and suppression of platelet adhesion (16). Vink and Duling (17) recently reported that the glycocalyx layer forms a selective barrier against penetration of macromolecules such as dextrans and proteins. In high-oxidative-stress models, the glycocalyx layer is degraded (16), and it is well known that in high-oxidative-stress conditions such as hypertension and diabetes mellitus, flow-dependent vasodilation by NO production is impaired (3, 14). Thus it is physiologically and pathophysiologically important to clarify shear detection mechanisms for flow-induced NO production.

Pohl et al. (13) studied the interactions of endothelium-derived NO and myogenic constriction in saline-perfused rabbit mesenteric arteries and reported that vasodilation by stepwise flow increase was converted to vasoconstriction after biodegradation of sialic acid glycosaminoglycans within the glycocalyx by neuraminidase. Hecker et al. (8) reported that neuraminidase treatment inhibited shear stress-dependent NO production in rabbit femoral arteries that were perfused at a constant flow rate. Hyaluronic acid glycosaminoglycans (hyaluronan) are another main component of the glycocalyx layer. However, the roles of hyaluronic acid glycosaminoglycans for endothelial function have been investigated only partially, e.g., regulation of permeation of macromolecules from plasma to the endothelial surface (10). It is well known that one of the major endothelial functions is NO production, and we recently measured (12) electrochemically the intravascular NO level of isolated canine femoral arteries perfused at a wide range of perfusion rates and observed a linear increase in the NO level with increasing perfusion rate. In this study, we aimed at measuring flow-induced NO production from isolated arteries at a range of perfusion rates before and after degradation of hyaluronic acid glycosaminoglycans to clarify the role

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of hyaluronic acid glycosaminoglycans within the glycocalyx layer in the flow-sensing mechanisms for endothelium-derived NO production.

MATERIALS AND METHODS

Experimental procedures were approved by the Animal Research Committee of Kawasaki Medical School and conform with the standards for use of laboratory animals established by the Institute of Laboratory Animal Resources, National Academy of Sciences (US).

Chemicals. Modified Krebs-Henseleit bicarbonate buffer (in mM: 11 D-glucose, 1.2 MgSO₄, 12 KH₂PO₄, 4.7 KCl, 120 NaCl, 25 NaHCO₃, and 2.5 CaCl₂·2H₂O), hyaluronidase (hyaluronic acid hydrolase), ϵ -amino-*n*-caproic acid (plasmin inhibitor), and benzamidine hydrochloride (peptidase inhibitor) were purchased from Sigma (St. Louis, MO). BSA was supplied by Nacalai Tesque (Kyoto, Japan) and acetylcholine by Daiichi Pharmaceutical (Tokyo, Japan). Stock solutions of 1) hyaluronidase and 2) ϵ -amino-*n*-caproic acid and benzamidine hydrochloride were prepared with ultrapure water and frozen at -30°C until use. These stock solutions and BSA were mixed and diluted with Krebs buffer on the day of experiment (final concentrations: 14 μ g/ml for hyaluronidase, 6.6 mg/ml for ϵ -amino-*n*-caproic acid, 1.6 mg/ml for benzamidine hydrochloride, 10 mg/ml for BSA). All solutions were purged with a mixed gas of 20% O₂-5% CO₂-75% N₂ and were kept at 37 \pm 0.5°C and pH 7.4.

Animals and isolated vessel preparation. Adult mongrel dogs ($n = 31$) of either sex, weighing 10–32 kg, were initially sedated with ketamine (200 mg im) and anesthetized with pentobarbital sodium (30 mg/kg iv). Animals were then ventilated with a respirator pump (3–5 l O₂/min; model VS600, Instrumental Development, Pittsburgh, PA). These dogs were used mainly for other experimental studies on coronary circulation, and we isolated femoral arteries for the present study. A 6-cm-long segment of femoral artery (outer diameter 2.2–4.5 mm in situ) was isolated from each animal. Immediately after isolation, each artery was flushed with a heparinized saline solution, cannulated with a silicone tube through a plastic connector, and then placed in a bath containing Krebs buffer (37 \pm 0.5°C) with the vessel length being kept to 6 cm (in vivo length). Here the proximal side of the vessel was connected to solution chambers and the distal side to a short tube for draining (sample collection for flow and nitrite measurements). These vessels were studied in a passive state without a constrictor, and also there was no spontaneous tone. The diameter was therefore constant throughout the experiment [confirmed by observation with a charge-coupled device (CCD) camera attached to a microscope].

Experimental protocol. First, the vessels were perfused with Krebs buffer within a wide range of perfusion rates (1.0–159.7 ml/min) to evaluate the control level of flow-induced NO production. Second, active hyaluronidase ($n = 11$) or heat-inactivated hyaluronidase ($n = 10$) was perfused for 20 min and then washed out with Krebs buffer for 10 min, and flow-induced NO production was measured again by perfusing Krebs buffer. Estimated shear stress ranged from 0.01 to 0.82 Pa (0.1–8.2 dyn/cm²). Here we followed previous protocols for hyaluronidase treatment (5, 15). Heat inactivation of hyaluronidase was performed by immersing the hyaluronidase stock solution in warm (90°C) water for 30 min. ϵ -Amino-*n*-caproic acid and benzamidine hydrochloride were not heat inactivated for both active and heat-inactivated hyaluronidase studies, to evaluate the sole effect of hyaluronidase treatment.

To confirm intactness of the endothelium and investigate the effect of hyaluronidase treatment on agonist-induced NO production from isolated canine femoral arteries ($n = 10$), acetylcholine was perfused at a constant perfusion rate before and after hyaluronidase treatment. First, 10 nM acetylcholine was perfused for 4 min to evaluate agonist-induced NO production by measuring the nitrite concentration in the perfusate and then washed out with Krebs-Henseleit buffer solution for 5 min before active hyaluronidase was perfused for 20 min to degrade the hyaluronic acid. After 10-min washout with Krebs buffer solution, acetylcholine was perfused again to evaluate agonist-induced NO production after degradation of hyaluronic acid. In each vessel, the perfusion rate was kept constant throughout the whole protocol to exclude possible effects of changes in shear stress on acetylcholine-induced NO production rate. Flow rate ranged from 16.2 to 21.7 ml/min (estimated shear stress: 0.06–0.07 Pa, 0.6–0.7 dyn/cm²).

Evaluation of NO production rate. Endogenous NO production rate was evaluated as the product of perfusion rate and nitrite concentration in the effluent. Perfusion rate was determined by timed collection; the weight of the effluent collected in a vial was measured by an electronic balance. About 200 μ l of the effluent solution was collected after 3 min at each perfusion rate for measurement of nitrite concentration in the effluent. Nitrite was measured by a NOx analyzer (ENO-20; Eicom, Kyoto, Japan). The minimum detectable concentrations are <5 nM in pH-neutral solutions. In this study, we chose nitrite measurement instead of NO measurement by an NO sensor because of the high stability and easy analysis of nitrite in an aqueous solution. In preliminary studies, NO production rate of an isolated canine femoral artery did not change significantly in three or four runs of perfusion, indicating no changes in the relation between perfusion rate and NO production rate during the experimental period of each vessel.

Data analyses. Linear regression and correlation analyses were applied to evaluate the slope of the linear relation between perfusion rate and NO production rate. Changes in flow-induced NO production were evaluated as changes in the slope (normalized by control). Statistical analyses for the normalized values of the slopes and of acetylcholine-induced NO (nitrite) production were conducted by paired (control vs. after treatment with active or inactivated hyaluronidase; before and after hyaluronidase treatment for acetylcholine studies) or unpaired (after active hyaluronidase vs. after inactivated hyaluronidase) *t*-test. A value of $P < 0.05$ was considered statistically significant. Values are means \pm SD.

RESULTS

The outer diameter of each vessel was constant throughout the experiment as confirmed by observation with a CCD camera attached to a microscope (data not shown). Perfusion pressure was <8 cmH₂O at the maximum perfusion rate for each vessel, indicating that in this study we observed the sole effect of shear stress by excluding the luminal pressure effect.

Figure 1 shows a representative result for flow-induced NO production before and after treatment with active hyaluronidase. The NO production rate increased linearly with increasing perfusion rate before enzymatic treatment ($r^2 = 0.95$). Hyaluronidase treatment significantly attenuated flow-induced NO production (decrease in slope from 0.084 to 0.009 nmol/ml). A linear relation between NO production rate and perfu-

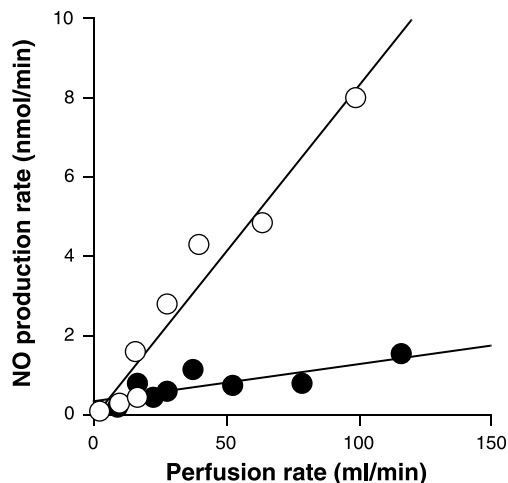


Fig. 1. Relation between nitric oxide (NO) production rate from the isolated canine femoral artery and perfusion rate before (\circ) and after (\bullet) treatment with $14 \mu\text{g/ml}$ hyaluronidase. Solid lines are a linear regression for each protocol (before: $y = 0.084x - 0.055$; after: $y = 0.009x + 0.374$).

sion rate was also observed after enzymatic treatment ($r^2 = 0.63$).

Figure 2 shows a representative result for flow-induced NO production before and after treatment with heat-inactivated hyaluronidase. In sharp contrast to active hyaluronidase, treatment with heat-inactivated hyaluronidase did not cause any changes in flow-induced NO production. A linear relation between NO production rate and perfusion rate was again observed before and after enzymatic treatment ($r^2 = 0.99$ for both).

Each data set, i.e., before or after enzymatic treatment, was then fitted with a linear regression line by the least-squares method ($r^2 = 0.63$ – 0.99), and the slope obtained was used as a measure of flow-induced NO production. Figure 3 summarizes the normalized

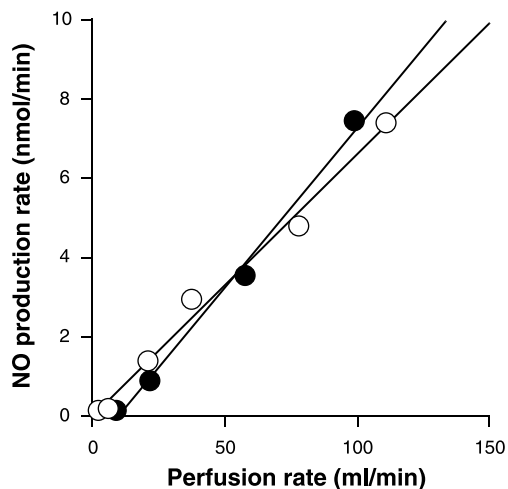


Fig. 2. Relation between NO production rate from the isolated canine femoral artery and perfusion rate before (\circ) and after (\bullet) treatment with $14 \mu\text{g/ml}$ heat-inactivated hyaluronidase. Solid lines are a linear regression for each protocol (before: $y = 0.066x + 0.019$; after: $y = 0.081x - 0.779$).

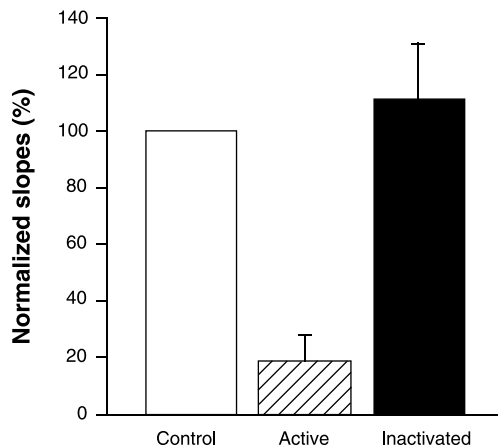


Fig. 3. Summary of the slopes of the linear correlation between perfusion rate and NO production rate. All values were normalized based on the slope before treatment (control, 100%). Control (open bar), after treatment with active hyaluronidase (hatched bar; $P < 0.0001$ vs. control; $n = 11$), and after treatment with heat-inactivated hyaluronidase [filled bar; $P =$ not significant (NS) vs. control, $P < 0.0001$ vs. active hyaluronidase; $n = 10$] values are shown.

slopes of the linear regression lines before and after treatment with active or heat-inactivated hyaluronidase. Here, the slope before treatment was used as control (100%). The normalized slope after active hyaluronidase treatment decreased significantly ($19 \pm 9\%$ of control; $P < 0.0001$ vs. control; $n = 11$), whereas that after treatment with heat-inactivated hyaluronidase did not change [$112 \pm 19\%$ of control; $P =$ not significant (NS) vs. control, $P < 0.0001$ vs. active hyaluronidase; $n = 10$].

Figure 4 summarizes the normalized nitrite concentration in the perfusate after 4-min acetylcholine perfusion before and after treatment with active hyaluronidase. Here, the nitrite concentration before hyaluronidase treatment was used as control (100%) for normalization, because vessel size widely ranged from 3 to 4 mm in outer diameter, with possible variations in NO production among the vessels. Hyaluronidase

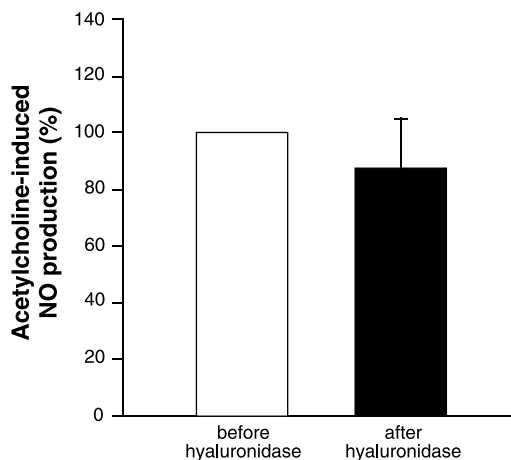


Fig. 4. Summary of effect of hyaluronidase treatment on acetylcholine-induced NO production ($n = 10$). All values were normalized based on nitrite concentration before treatment. Pretreatment (open bar, 100%) and posttreatment (filled bar; $P =$ NS) levels are shown.

treatment did not change acetylcholine-induced NO production significantly ($88 \pm 17\%$ of pretreatment level; $P = \text{NS}$; $n = 10$). Acetylcholine-induced NO production rate excluding shear-induced NO was 0.6–2.7 nmol/min. This range of NO production rate for 10 nM acetylcholine is comparable to the flow-induced NO production rate at the perfusion rate applied.

DISCUSSION

The present study showed that degradation of hyaluronic acid glycosaminoglycans within the glycocalyx layer significantly decreases flow-induced endothelial NO production in canine femoral arteries, indicating that hyaluronic acid glycosaminoglycans are essential elements for detection and amplification of flow-induced shear force toward the endothelial cell membrane.

In this study, flow-induced NO production was studied within a wide range of perfusion rates (1.0–159.7 ml/min; estimated shear stress: 0.01–0.82 Pa, 0.1–8.2 dyn/cm²) and ~80% NO production was inhibited by hyaluronidase treatment. Previously, Pohl et al. (13) investigated the role of sialic acid glycosaminoglycans of the rabbit mesenteric arteries and reported that neuraminidase treatment converted a net dilation to a net constriction when flow was increased ~2.5 times control. Hecker et al. (8) studied the role of sialic acid glycosaminoglycans of rabbit femoral artery segments in flow-induced NO production at a constant perfusion rate of 0.17 ml/min and observed 64% inhibition after neuraminidase incubation. Thus it was demonstrated that not only sialic acid but also hyaluronic acid within the glycocalyx layer plays a pivotal role in flow-induced NO production. Here, a minimal amount of flow-induced NO production was still observed after hyaluronidase treatment. Recently, Van den Berg et al. (15) observed marked but incomplete removal of the glycocalyx layer of the rat myocardial capillary endothelium after hyaluronidase treatment by transmission electron microscopy. Therefore, the remaining low degree of NO production may have reflected incomplete degradation of hyaluronic acid glycosaminoglycans within the glycocalyx layer and/or residual shear-detection mechanisms including other glycocalyx components, membrane fluidity, and cytoskeleton structure as discussed below. It is also interesting to note that the linear relation between NO production rate and perfusion rate was still observed even after hyaluronidase treatment.

The results for acetylcholine-induced NO production imply that hyaluronic acid is involved only in shear-induced NO production and not in agonist-induced NO production. This is similar to the role of sialic acid glycosaminoglycans within the glycocalyx as observed by Pohl et al. (13).

Change in shear stress due to a change in anatomic inner vessel diameter by glycocalyx degradation is negligibly small for the type of vessels used in this study. For example, complete degradation of a glycocalyx layer of 0.2 μm thickness [observed by Henry and

Duling (10) with FITC-dextran and a fluorescence microscope and by Van den Berg et al. (15) with a transmission electron microscope] of a vessel of 3-mm inner diameter would lower shear stress by only 0.04%. This small change in shear stress is not expected to cause such a large change in NO production (12). Thus it is considered that the glycocalyx layer is directly involved in the enhanced shear detection for NO production.

The exact shear detection mechanism of the glycocalyx layer is still speculative. As extensively discussed by Henry and Duling (10), hyaluronic acid glycosaminoglycans seem to create a meshlike matrix by spanning other molecules such as glycoproteins and proteoglycans. Van den Berg et al. (15) recently observed the glycocalyx meshlike structure and degradation of the structure after hyaluronidase treatment in their transmission electron microscopy studies. Accordingly, it is speculated that the shear effect is enhanced by this meshlike structure and that degradation by hyaluronidase treatment thus creates a more open structure, decreasing shear force on this cell surface structure.

The signal transduction mechanisms between glycocalyx and NO synthase (NOS) are not yet clarified. In light of several previous studies, the following mechanisms may be hypothesized. Activation of pertussis toxin-refractory G proteins is needed for the first phase of shear-induced activation of endothelial NOS (11). Gudi et al. (6) reported that activation of G proteins is dependent on the fluidity of the phospholipid bilayer of liposomes. Interestingly, the membrane fluidity of cultured human umbilical vein endothelial cells increased linearly with increasing shear stress (7). Butler et al. (2) also studied the changes in endothelial cell membrane fluidity by changing shear stress and observed time-dependent and spatially heterogeneous changes in the membrane fluidity. Because glycosaminoglycans are associated with cell membrane and membrane proteins, it is thus conceivable that the glycocalyx layer senses and amplifies the shear effect, leading to changes in membrane fluidity, G protein activation, and NOS activation. Other possible shear-force transduction mechanisms include distortion and rearrangement of the cytoskeleton (4, 9). The enhanced shear effect by the glycocalyx may further enhance mechanical forces on the cytoskeleton structure, which is associated with membrane proteins and thus may interact with membrane fluidity. Therefore, we speculate that fluid shear is detected by the integrated mechanisms of these processes.

Vink et al. (16) recently reported that the glycocalyx of hamster cremaster muscle capillaries is transiently degraded after oxidized LDL infusion. It is thus implied that in high-oxidative-stress states such as hypertension and diabetes mellitus, the effect of shear stress stimuli is less than in normal states because of glycocalyx degradation, resulting in decreased NO production. This may be part of the mechanism for endothelial dysfunction in addition to NO deactivation with superoxide in high-oxidative stress subjects. In our preliminary study, we concomitantly perfused tiron,

a superoxide scavenger, with active hyaluronidase. There was no change in the degradation effect of the enzyme (data not shown). Thus superoxide was not involved in the effects of hyaluronidase.

In conclusion, the hyaluronic acid glycosaminoglycans in the glycocalyx layer function as a shear-stress detection mechanism for shear-induced NO production.

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DISCLOSURES

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