Hyaluronidase treatment of coronary glycocalyx increases reactive hyperemia but not adenosine hyperemia in dog hearts

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TO ASSESS the functional severity of coronary stenoses in the clinic, use of adenosine for inducing maximal coronary hyperemia in patients has been well established (8, 10, 17, 34). Clinical decision-making to intervene is based on intracoronary parameters that are derived during hyperemia, and it is therefore critical that the adenosine-induced hyperemia accurately reflects the coronary hyperemia that can be attained by endogenous stimuli (7). Intravital microscopic studies of cremaster tissue have suggested that adenosine affects capillary perfusion by limiting the effect of the glycocalyx on vascular resistance (3, 12, 21). The glycocalyx is the carbohydrate-rich matrix that lines the luminal surface of blood vessels and forms the true interface between the endothelium and flowing blood (6, 24, 26, 30). Normally, flowing red blood cells and large dextrans are excluded from the glycocalyx in cremaster capillaries (6, 30, 31). However, it was shown that adenosine superfusion impaired glycocalyx exclusion properties (21). Because an intact glycocalyx strongly reduces the volume available for flow of plasma and red blood cells in capillaries (3, 22, 30), an impaired glycocalyx exclusion during adenosine is consistent with an increase in functionally perfused capillary volume, and as a result, elevated capillary tube hematocrit (12).

The aim of the present study was to determine to which extent the glycocalyx limits coronary hyperemia obtained with adenosine compared with hyperemia obtained by an ischemic stimulus [reactive hyperemia (18)]. In anesthetized dogs, coronary pressure and flow were measured in the left circumflex artery, and reactive hyperemia was compared with the hyperemia induced by intracoronary injection of adenosine. To determine the influence of the glycocalyx, interventions were repeated after hyaluronidase treatment of the coronary vascular bed, which reduces glycocalyx structures and increases its porosity (6, 26). It was hypothesized that maximal coronary conductance obtained during reactive hyperemia would be lower than during adenosine hyperemia in control conditions but that this difference would be smaller in case of a degraded glycocalyx.

METHODS

Animal preparation. All procedures and protocols were approved by the Animal Care and Use Committee of the Academic Medical Center. Experiments were performed in 11 mongrel dogs (~25 kg body wt). At the beginning of an experiment, dogs were premedicated with an intramuscular injection of 1 ml methadone (10 mg/ml) and 2 ml xylazine (20 mg/ml). General anesthesia was induced by intravenous administration of 4 ml pentobarbital sodium (60 mg/ml). Dogs were intubated and ventilated by a Harvard respirator with a 2:1 N2O-O2 mixture. Anesthesia was maintained by intravenous administration of 40 ml fentanyl (0.05 mg/ml) in 30 min. After preparative surgery, the anesthesia for the experiments was continued by injection of 5 ml fentanyl and 2 ml of pentobarbital sodium per hour. Depth of anesthesia was controlled by checking reflexes and by monitoring heart rate and aortic pressure (\(P_{\text{aao}}\)). Arterial blood gasess and pH were measured every 30 min. When necessary, ventilation was adjusted to maintain oxygen and CO2 pressures within physiological limits, and sodium bicarbonate was administered to avoid acidosis.

The left carotid artery was cannulated for \(P_{\text{aao}}\) measurement and arterial blood sampling. A left thoracotomy was performed in the fifth intercostal space, and the heart was exposed and suspended in a pericardial cradle. A catheter tip manometer (Millar SPC-350) was inserted through the left atrial appendage into the left ventricular...
cavity for measurement of left ventricular pressure (Pv). The middle portion of the left circumflex artery (LCx) was dissected free, and a ligature and a 2-mm perivascular transit-time flow probe (type 2SB; Transonics System) were placed around the vessel. The flow probe was connected to a transit-time flowmeter (Transonic T206-S) for measurement of LCx blood flow (Q˙LCx). Distal to the flow probe, a small Venflon cannula (22G/0.8-mm OD, 25-mm length) was introduced in downstream direction in the artery for measurement of LCx pressure (Pc,LCx) and for intracoronary administration of adenosine and hyaluronidase. Heparin was not used in the experiments.

**Experimental protocol.** After surgery, the preparation was allowed to equilibrate for 20 min. Hyperemia in the LCx was induced in two ways: 1) by 15 s occlusion using the ligature [reactive hyperemia (RH)], and 2) by injection of adenosine (650 μg in 0.5 ml saline, diluted in 2 ml blood; infused in ~10 s) [adenosine-induced hyperemia (AH)]. This occlusion duration and adenosine dose were shown to induce maximal coronary hyperemia in the dogs as longer occlusions and higher doses could not further increase coronary conductance. RH and AH were alternately applied, and hemodynamic parameters were allowed to recover after each hyperemia. As such, a paired RH and AH intervention was performed within a 10- to 15-min period. In each animal, the following protocol was applied after the equilibration period. First, RH and AH control interventions were performed (Con). Subsequently, hyaluronidase (40 ml of 500 U/ml in Haemacell) was infused for 20 min, and interventions were performed for 45 min after the infusion (HYAL1). To aggravate the glycoscalyx degradation (26), a second 20-min infusion of hyaluronidase was then given, and RH and AH interventions were again performed up to 45 min after the infusion (HYAL2). Active hyaluronidase (type IV-S, Sigma) was infused in n = 6 animals; the other animals (n = 5 animals) served as control group and received heat-denatured hyaluronidase (95°C for 10 min).

**Measurements and data analysis.** Zero pressures were set at mid-chest level. Zero flow was obtained during LCx occlusion. Throughout the experiment the following variables were recorded continuously: Pw, Pc,LCx, and PLCx. Signals were digitized and stored on a personal computer for off-line analysis. Heart rate was determined from Pw. Baseline values were determined as average of 10 subsequent heartbeats before an intervention, and hyperemic values were determined as average of three heartbeats during peak coronary flow. LCx conductance (CCLCx) was calculated as Q˙LCx/Pc,LCx. The difference between hyperemic CCLCx during RH vs. AH was quantified by determining their ratio for each paired RH and AH. Repeated observations during a given condition (Con, HYAL1, HYAL2) were averaged. Time-dependent effects of hyaluronidase were analyzed using repeated-measures analyses of variance; in case of significant main effects, post hoc comparisons were performed using Tukey tests. Differences between RH vs. AH regarding Q˙LCx and CCLCx within a given condition were tested with paired t-tests. Results were considered statistically significant with P < 0.05. Summary data are reported as means ± SE.

**Table 1. Baseline hemodynamic data**

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<tr>
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<th>Active Hyaluronidase</th>
<th>Heat-Inactivated Hyaluronidase</th>
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<tr>
<td></td>
<td>Control</td>
<td>HYAL1</td>
</tr>
<tr>
<td>Pw, mmHg</td>
<td>106±8</td>
<td>101±10</td>
</tr>
<tr>
<td>Pcw, mmHg</td>
<td>40.9±6.5</td>
<td>40.4±4.7</td>
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<tr>
<td>Heart rate, beats/min</td>
<td>81±6</td>
<td>81±4</td>
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<tr>
<td>PLCx, mmHg</td>
<td>104±7</td>
<td>97±9*</td>
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<tr>
<td>Q˙LCx, ml/min</td>
<td>26.9±3.2</td>
<td>31.6±3.8</td>
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Values are means ± SE. Pw, left ventricular cavity pressure; HYAL1, after 1st infusion of hyaluronidase; HYAL2, after 2nd infusion of hyaluronidase. After infusion of active and heat-inactivated enzyme there was a decrease (one-way repeated measures ANOVA, main effects) in aortic pressure (Pw) (both P < 0.05) and left circumflex pressure (PLCx) (both P < 0.01) and increase (one-way repeated measures ANOVA, main effects) in left circumflex blood flow (Q˙LCx) (P < 0.05 active, P < 0.01 inactivated enzyme). *P < 0.05 compared with control.

**RESULTS**

Baseline hemodynamic parameters are presented in Table 1. In both hyaluronidase-treated animals as well as control animals that received the denatured enzyme, mean Pw and PLCx decreased and Q˙LCx increased after infusions. Mean Pw and heart rate did not change after respective infusions.

Examples of flow responses in the LCx following RH and after adenosine injection (AH) are shown in Fig. 1. The left panels demonstrate the control condition and the right panels the condition after the second infusion of hyaluronidase. After release of the 15-s occlusion, blood flow increased and attained a peak after 5–10 s (peak RH flow). During control conditions this peak RH flow was lower than the peak flow obtained after adenosine. After hyaluronidase infusion, however, peak RH flow increased compared with control and appeared no longer different from AH.

In Fig. 2, average data of PCLCx and Q˙LCx during RH and AH during control conditions and after the first (HYAL1) and second (HYAL2) infusion of active vs. heat-inactivated hyaluronidase are presented. Consistent with the decrease in baseline aortic and coronary pressures, hyperemic PCLCx decreased with ~15 mmHg after infusion of active and heat-inactivated hyaluronidase but was not different between RH and AH. Hyperemic Q˙LCx did not change after active hyaluronidase but decreased in the dogs that received denatured hyaluronidase. During control conditions, Q˙LCx was lower during RH than AH (127 ± 11 vs. 161 ± 11 ml/min; pooled groups). A difference between RH and AH flow remained after the second infusion of heat-inactivated hyaluronidase (101 ± 13 vs. 131 ± 14 ml/min) but was not present anymore in the active enzyme group (156 ± 15 vs. 165 ± 15 ml/min). Heart rate and Pw did not change during interventions or enzyme infusions.

Hyperemic CCLCx is presented in Fig. 3 (left, active enzyme; right, inactivated enzyme). During control conditions, conductance during RH was 1.49 ± 0.15 ml·mmHg⁻¹·min⁻¹ and lower than conductance during AH (1.95 ± 0.16 ml·mmHg⁻¹·min⁻¹; pooled groups). Without a change in AH conductance, RH conductance increased by 28 ± 14 (HYAL1) and 43 ± 13% (HYAL2) of control after the two subsequent infusions of active hyaluronidase. The ratio between CCLCx during RH vs. during AH increased from 0.76 ± 0.07 in control to 0.87 ± 0.02 and 0.93 ± 0.04 after the first and second hyaluronidase infusion. After the second infusion, RH conductance did not differ anymore from AH conductance. In contrast to active hyaluronidase, infusion of heat-inactivated hyaluronidase had
Fig. 1. Representative tracings of coronary pressure and blood flow during reactive hyperemia (RH) and adenosine bolus. Top: left circumflex pressure ($P_{LCx}$). Bottom: left circumflex blood flow ($Q_{LCx}$). Left: control conditions. Right: after second infusion of active hyaluronidase. Occlusion of left circumflex (LCx) inflow starts at time = 0 s; $Q_{LCx}$ becomes 0 and $P_{LCx}$ drops to coronary wedge pressure ($P_w$). Baseline data before adenosine administration did not differ from baseline data before RH and are not shown.

Fig. 2. Effect of hyaluronidase treatment on coronary pressure and flow during RH and adenosine hyperemia (AH). Top: $P_{LCx}$. Bottom: $Q_{LCx}$. Left: active hyaluronidase. Right: heat-inactivated hyaluronidase. HYAL1, after 1st infusion of hyaluronidase; HYAL2, after 2nd infusion of hyaluronidase. $P_{LCx}$ decreased after infusion of active and heat-inactivated enzyme (both groups $P < 0.01$; 2-way repeated measures ANOVA, main effects); the decrease did not differ between AH and RH interventions. $Q_{LCx}$ did not change in the animals that received active hyaluronidase but decreased in the ones that received heat-inactivated enzyme ($P < 0.01$; 2-way repeated measures ANOVA, main effect); in both groups $Q_{LCx}$ differed between RH and AH (both $P < 0.05$; 2-way repeated-measures ANOVA, main effects). During control conditions, $Q_{LCx}$ was lower during RH than AH ($P < 0.05$, paired t-test). A difference remained after the second infusion of heat-inactivated hyaluronidase but not after the second infusion of active enzyme. *$P < 0.05$ compared with control. #$P < 0.05$ compared with $Q_{LCx}$ during AH.
no effect on $C_{LCx}$ during RH or AH and the ratio of the former to the latter.

**DISCUSSION**

The current study in dog hearts shows that the glycocalyx limits coronary hyperemia after an ischemic stimulus of 15 s but has hardly an effect on AH. Intracoronary hyaluronidase infusion did not affect coronary conductance obtained with adenosine but increased coronary conductance during RH by $\sim 40\%$ and resulted in near equal conductances during RH and AH. Therefore, when glycocalyx function is intact, AH as a clinical test may considerably overestimate the coronary ability to adapt to an increase in myocardial oxygen demand.

**Methodological considerations.** We deliberately did not use a perfusion system in the current study to avoid removal of the endothelial glycolcayx by hemodilution with artificial media (23). To degrade the glycocalyx in the coronary circulation, we infused hyaluronidase at a concentration of 500 U/ml in the LCx region for two subsequent 20-min periods. With a $Q_{LCx}$ of $\sim 30$ ml/min (Table 1) and infusion rates of 2 ml/min, the estimated concentration in the coronary circulation is comparable to the 25 U/ml that was used previously by van den Berg et al. (26) in isolated rat hearts. In that study, the endothelium-associated carbohydrate layer in capillaries, visualized by electron microscopy, was greatly reduced by 1-h perfusion with hyaluronidase in the medium and resulted in significant myocardial interstitial edema formation (26). The in vivo beating heart is less susceptible to interstitial edema formation because cardiac contraction constitutes an important defense mechanism against increases in interstitial fluid (29). Hemodynamic parameters were modestly altered after hyaluronidase and comparable to the changes in the animals that received the inactivated enzyme (Table 1), suggesting that cardiac function was not affected by hyaluronidase per se in the present study.

**RH vs. AH.** In contrast to the decrease in $Q_{LCx}$ after administration of heat-inactivated enzyme, hyperemic coronary blood flow was not diminished after active hyaluronidase despite the equal decrease in $P_{LCx}$ (Fig. 2). To account for the decrease in coronary pressures during the course of the experiments, $C_{LCx}$ was calculated (Fig. 3). During control conditions, coronary conductance after adenosine exceeded that during peak RH, resulting in a ratio of the latter to the former of 0.76 ± 0.07. Several previous studies in dogs have compared peak coronary hyperemia after occlusion vs. hyperemia induced by intravenous or intracoronary administration of pharmacological agents. These studies have reported similar results between the two interventions (4, 19), as well as higher values during RH (9) or higher values with vasodilators (1, 11, 33). Differences in agents, concentrations, and route of administration seem to underlie the reported disparity. The amount of adenosine given in the current study was 650 $\mu$g, a dose that in our laboratory was found necessary to induce maximal coronary hyperemia in dogs. This dose is much higher than the 20–40 $\mu$g that is typically used in patients. Comparison of concentrations between human and animal studies is ambiguous, however, because it is suggested that humans are more sensitive to the vasodilator properties of adenosine or have a slower rate of elimination of adenosine than dogs (34). Indeed, intracoronary administration of adenosine at doses used in humans was found to elicit only submaximal hyperemia in dogs (9). Although the use of milligram doses of adenosine in the clinic has been advocated (14), our data suggest that application of such high adenosine concentrations might increase the discrepancy between pharmacologically induced dilation vs. ischemia-induced hyperemia.

**Hyaluronidase effects on glycocalyx and coronary conductance.** $C_{LCx}$ during peak RH increased to $\sim 140\%$ of control after the second infusion of the active enzyme (Fig. 3). The
increase in conductance likely reflects an increase in coronary vascular volume available for blood flow. Pries et al. (1997) calculated that effective vessel diameter available for red blood cell and plasma motion had increased by at least 0.35–0.55 μm after a single ~10 min infusion of heparinase in the rat mesentery bed. Indeed, a glycocalyx with these dimensions is typically reported in capillaries of cremaster tissue (6, 21, 30, 31), and has been suggested to be present in myocardial capillaries as well (26). Pries et al. (22) found a 14–21% decrease in microvascular flow resistance after enzyme treatment. Our data fit well with these results because the increase in conductance that we observed after the second infusion of hyaluronidase corresponds with an actual decrease in coronary resistance of 27 ± 7% (data not shown).

In contrast to the increase in coronary conductance during RH, glycocalyx degradation did not affect maximal conductance in response to adenosine infusion, indicating that the microvascular resistance offered by the glycocalyx is reduced during AH. These results might be explained by an increased accessibility of the glycocalyx by circulating blood as suggested by intravital microscopic observations of an impaired ability of the glycocalyx to exclude 70-kDa FITC-dextran and red blood cells during adenosine superfusion in cremaster capillaries (21). The effect of adenosine on the glycocalyx does not necessarily have to reflect a true physical degradation of this layer. Rather, an increase in glycocalyx porosity might be involved because the decrease in exclusion of dextrans was found to be much more profound, and to occur at lower concentrations, than that of red blood cells (21). In a previous study of the Duling group, systemic administration of hyaluronidase was equally found to increase glycocalyx porosity independently from a change in the physical dimensions of the layer as judged from the exclusion of red blood cells (6). The selective increase in porosity of the glycocalyx might be indicative of an increased plasma flow through this layer, thereby explaining the increase in capillary tube hematocrit during adenosine (12). This increase in functionally perfused capillary volume might underlie the mismatch that has been reported between increases in estimated epicardial capillary volumetric flow and coronary arterial flow during AH but not RH (11).

The idea that adenosine-induced coronary hyperemia includes vasodilation of the capillary compartment is substantiated by previous studies (13, 27). Using hemoglobin-bound oxygen as endogenous tracer, Van der Ploeg et al. (27) estimated total capillary volume to increase by 47 ± 14% during maximal vasodilation with adenosine in goat hearts. Using CT, Liu et al. (13) found a substantial increase in intramyocardial blood volume with an increase in blood flow during adenosine in pig hearts. These authors estimated capillary volume to increase linearly with flow, based on the suggestion that increases in perfused capillary volume during adenosine were brought about by the recruitment of capillaries that previously did not participate in flow conductance. It should be anticipated, however, that increases in perfused capillary volume by an increased permeability of the glycocalyx can occur at the level of the individual capillary.

Clinical implications. For evaluation of the functional severity of coronary stenoses in the clinic (5, 20, 25), adenosine-induced coronary hyperemia is taken as index for the maximal obtainable conductance induced by increased cardiac work or an ischemic stimulus. In the present study in dog hearts, maximal conductance during peak RH was found to be ~25% lower than that obtained during adenosine and the difference was almost gone after treatment of the glycocalyx. Use of a high dose of adenosine in the clinic can therefore result in an overestimation of true coronary flow reserve in case of an intact glycocalyx. In patients with coronary artery disease (CAD), the glycocalyx might well be affected, in particular in those patients in which endothelial dysfunction is manifest. In these patients, the overestimation of flow reserve with adenosine may thus be diminished. The glycocalyx is vulnerable to atherogenic conditions (32), and its degradation results in an impaired NO production in response to shear stress (16) as well as increases in vascular permeability (28) and adhesiveness (2, 32). Changes in glycocalyx integrity thus influence microvascular resistance by affecting perfused capillary volume as well as endothelial function. Microvascular resistance plays a prominent role in the outcome of intracoronary parameters in patients with intermediate coronary lesions (15), and it would be of value to measure the condition of the coronary glycocalyx in these CAD patients. As such, our current research efforts are aimed at developing the methodology to measure coronary glycocalyx volume in patients.

In conclusion, we demonstrate in the current study that, compared with RH, adenosine-induced coronary hyperemia is barely limited by the glycocalyx. This glycocalyx insensitivity should be taken into account when using adenosine-induced coronary hyperemia as marker for maximum vasodilating capacity to an ischemic stimulus.

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GRANTS

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REFERENCES

CORONARY GLYCOCALYX AND FLOW RESERVE


