Reactive oxygen species mediate modification of glycocalyx during ischemia-reperfusion injury

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Reactive oxygen species mediate modification of glycocalyx during ischemia-reperfusion injury. Am J Physiol Heart Circ Physiol 290: H2247–H2256, 2006. First published January 6, 2006; doi:10.1152/ajpheart.00796.2005.—The glycocalyx (Gcx) is a complex and poorly understood structure covering the luminal surface of endothelial cells. It is known to be a determinant of vascular rheology and permeability and may be a key control site for the vascular injuries caused by ischemia-reperfusion (I/R). We used intravital-microscopy to evaluate the effects of I/R injury on two properties of Gcx in mouse cremaster microvessels: exclusion of macromolecules (anionic-dextrans) and intracapillary distribution of red blood cells (RBC). In this model, the Gcx is rapidly modified by I/R injury with an increase in 70-kDa anionic-dextran penetration without measurable effect on the penetration of 580-kDa anionic-dextran or on RBC exclusion. The effects of I/R injury appear to be mediated by the rapid production of reactive oxygen species (ROS) because they are ameliorated by the addition of exogenous superoxide dismutase-catalase. Intravenous application of allopurinol or heparin also inhibited the effects of I/R injury, and we interpret efficacy of allopurinol as evidence for a role for xanthine-oxidoreductase (XOR) in the response to I/R injury. Heparin, which is hypothesized to displace XOR from a heparin-binding domain in the Gcx, reduced the effects of I/R. The effects of I/R injury were also partially prevented or fully reversed by the intravascular infusion of exogenous hyaluronan and heparin. These data demonstrate: 1) the liability of Gcx during I/R injury; 2) the importance of locally produced ROS in the injury to Gcx; and 3) the potential importance of heparin-binding sites in modulating the ROS production. Our findings further highlight the relations between glycosaminoglycans and the pathophysiology of Gcx in vivo.

glycosaminoglycans; heparin-binding domain; xanthine oxidoreductase

THE LUMINAL ENDOTHELIAL GLYCOCALYX (Gcx) was once viewed as a thin, simple matrix first visualized by ruthenium red deposition in electron micrographs. However, in the intact vasculature, it is now known to be a large and complex structure dependent on the presence of abundant glycosaminoglycans (GAGs) (50, 51). The abundant Gcx appears to play a critical role in several endothelial cell functions, including modulation of transcapillary oncotic forces (28, 29), endothelial permeability (24), and hydraulic conductivity (23, 56). In addition, the Gcx prevents direct interactions between the endothelial surface and red and white blood cells as well as plasma proteins (10, 13, 14, 32, 57). Moreover, it has recently been proposed that the Gcx is a transducer between cell surface mechanical forces and endothelial signaling (52, 53, 68).

The complexity of the endothelial cell Gcx is emphasized by the recognition that it has the ability to selectively bind a variety of proteins through a heparin-binding domain (HBD), and these proteins may play key roles in the regulation of endothelial cell function (see reviews in Refs. 7 and 41). Among the known Gcx-bound proteins are such vital enzymes as xanthine oxidoreductase (XOR; see Refs. 1 and 36), superoxide dismutase (SOD) (2), and clotting factors such as antithrombin III (58), apolipoproteins (54), selectins (35), and chemokines (e.g., IL-8 and MCP-1) (27).

The Gcx has long been recognized as a site of damage following a period of ischemia (ischemia-reperfusion injury, I/R-I) (22), and endothelial cells of the microvasculature are especially vulnerable (21) perhaps as a result of production of reactive oxygen species (ROS) (4, 43). A critical role for ROS in I/R-I has recently been supported by a reported protective effect of overexpression of SOD in transgenic mice (8, 42). Sources of ROS are likely to include activated leukocytes (33, 39), vascular NADPH oxidase (16), and XOR (5, 36).

From the demonstration that hyaluronan is present in the Gcx (24) and the well-known sensitivity of this molecule to ROS, hyaluronan may be a likely target for ROS (39), leading to hyaluronan fragmentation and the production of lower molecular weight chains of hyaluronan, which could intensify edema and impair healing (38). Moreover, in studies where the integrity of the hyaluronan in the Gcx was compromised by hyaluronidase, pathophysiological alterations were similar to those induced by I/R-I (24, 62). Interestingly, Henry and Duling (24) showed that an effect of hyaluronidase on the Gcx could be reversed by the addition of exogenous hyaluronan and chondroitin sulfate, suggesting that a similar treatment might reverse the effects of I/R-I.

Based on the preceding data, we hypothesized that I/R-I in the microvasculature would alter the luminal endothelial Gcx via ROS. Furthermore, we hypothesized that ROS are generated in part by XOR bound to the Gcx through its HBD and that infusion of exogenous hyaluronan would prevent or reverse the I/R injury-induced effects. We have tested these hypotheses in the microvasculature of the mouse cremaster.

MATERIALS AND METHODS

Animal preparation. The Institutional Animal Care and Use Committee at University of Virginia approved all protocols and animal-

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 handling procedures. Male C57BL/6 mice (20–25 g) were anesthetized with an intraperitoneal injection of pentobarbital sodium (40 mg/kg). The right femoral vein was cannulated and maintained patent with sterile physiological saline solution (0.9% sodium chloride solution). A detailed description of the cremaster muscle preparation for in vivo microscopy was published previously (24, 64). After 45 min of stabilization, arteriolar responses to 10% O2 and acetylcholine (20 μmol/l) were ascertained to assess arteriolar reactivity and tone, following which a fluorescent anionic dextran (0.05 ml of 20 mg/ml) was administered intravenously.

Intravitral microscopy. Capillaries and postcapillary venules were randomly selected for observation with a ×60 (numerical aperture 0.9) water immersion objective. A 150-W xenon lamp provided the source for brightfield measurements, and fluorescence epi-illumination was obtained from a 75-W xenon lamp equipped with 450- to 490-nm and 510- to 560-nm excitation filters, dichroic beam splitters (FT510 and 580), and barrier filters (LP520 and 590). Epi-illumination of each vessel was limited to <10 s to prevent light-dye injury to microvessels (65). Brightfield and epi-fluorescence images of vessels were displayed on a video monitor and recorded at 5-min intervals on S-VHS videotapes for subsequent analysis.

Image analysis. Recorded video images of the experiments were analyzed with Image-1 software (Universal Imaging). For each vessel, the anatomic diameter (brightfield microscopy), the width of the fluorescent dextran column (epi-fluorescence microscopy), and the average diameter of the red blood cell (RBC) column (brightfield microscopy, 420-nm filter) were measured along a 5.3-μm segment of the vessel by using calibrated video calipers. As previously reported (24, 25, 47, 48, 64, 65), neither the dextran nor the RBC reached the surface of the endothelial cell as defined in brightfield microscopy. The widths of RBC and Dex-70-FITC, Dex-580-FITC and carboxymethylamino-dextran-70 (CMADR)-70 exclusion zones were calculated as the difference between the brightfield capillary diameter and diameters of the RBC and Dex70-FITC, Dex 580-FITC, and CMADR-70 column divided by two (47, 48, 64).

I/R model. A 20-min baseline was established before ischemia or sham treatment. Global ischemia of the left cremaster was induced by cross clamping the pedical of the cremaster with an atrumatic vascular clamp (48) thereby occluding all afferent and efferent vessels of the cremaster. Ischemia was maintained for 45 min, and after the clamp was removed, reperfusion was confirmed by visualization of flow in the arteriolar and venular networks. Diameters of the capillary lumen, the RBC, and the dye columns were determined in selected capillaries and postcapillary venules observed at the time of release and at roughly 5-min intervals thereafter for 45 min. We performed sham occlusions by manipulating the tissue and placing the vascular clamp under the cremaster for 45 min.

Experimental protocols. To evaluate the effect of I/R on macro-molecular cutoff sizes of the matrix constituting the Gcx, we simultaneously infused two different sizes of fluorescent anionic dextrans (70 and 580 kDa, labeled with rhodamine and FITC, respectively). A bolus of either anionic dextran was infused, and a baseline was measured every 5 min for 25 min. Then a bolus of the other anionic dextran was infused, and the baseline for both dextrans was evaluated as above mentioned. Global ischemia and reperfusion or sham procedures were made as described previously.

Reduced dextran synthesis. Anionic dextran labeled with rhodamine (70 kDa) is not commercially available. Therefore, CMADR was synthesized as previously described (55). Briefly, Dex70 was carboxymethylated by reaction of the dextran with 1 mol/l chloroacetic acid in 3 mol/l NaOH for 1 h at room temperature. The reaction was neutralized to pH 7.0 and dialyzed for 5 days against distilled water. Ethylene diamine was conjugated to a limited number of carboxymethyl groups by adding N-hydroxysulfosuccinimide and 1-ethyl-3-(3-dimethylamino propyl)carbodiimide HCl at pH 7.4 for 2 h at room temperature. This reaction was stopped by ultrafiltration with Centrulplus centrifugal filter devices (cutoff 10,000 mol wt). Finally, N-hydroxy-sulfosuccinimide-rhodamine (5 mg/ml in DMSO) was linked to the carbamoylmethylamino-dextran by incubation in 50 mmol/l sodium bicarbonate buffer, pH 7.0, at 4°C for 2 h in the dark.

CMADR was concentrated and purified from unbound fluorescence with Centrulplus centrifugal filter devices (cutoff 10,000 mol wt). The relative charge was determined by isoelectric focusing (IEF) by using Criterion IEF gel electrophoresis, as described by the manufacturer (Bio-Rad). Briefly, IEF standards (phycocyanin pI = 4.6, bovine carbonic anhydrase = 6.0, equine myoglobin = 6.8 and 7.0, human hemoglobin A = 7.1, lentil lectin = 7.80, 8.00, and 8.20; cytochrome c = 9.6), CMADR-70, BSA-FITC, Dex70-FITC, and Dex 580-FITC were loaded on top of IEF gel, and the gel was exposed to stepwise power increase, starting at 100 V for 60 min, 250 V for 60 min, and 500 V for 30 min.

To evaluate the participation of ROS in the effect of I/R on the Gcx, a mixture of 50 units of SOD and 50 units of catalase (SOD/Cat) was administered intraperitoneally 3 min before the vascular clamp removal. At the same time, both enzymes were added to the superfusion solution (1.25 U/ml).

We evaluated the participation of two different sources of ROS in microvessels, NADPH oxidase, and XOR. XOR was evaluated by intravenous administration of allopurinol, an inhibitor of XOR (1,5-dihydro-4H-pyrazolo[3,4-d]pyrimidin-4-one, 0.5, 2.5, or 5 mg/kg) 5 min before vascular clamp removal. At the same time, allopurinol was added to the superfusion solution (2.5, 25, or 250 μmol/l) and continued until the end of the experiment. Diphenyleneiodonium (DPI) was used to evaluate the role of NADPH oxidase as the source of ROS. A single dose of DPI (1 mg/kg) was administered intravenously 5 min before reperfusion, at the same time the superfusion solution was added with DPI to a final concentration of 5 μmol/l as noted above. In separate experiments, heparin (300 U/kg) was administered intravenously 3 min before the onset of reperfusion in an effort to displace molecules such as extracellular SOD and XOR, which are bound to sulfate-GAGs through a HBD (1, 2).

To assess the ability of the GAGs to modify the course of the I/R-I in the Gcx, we also infused hyaluronan and chondroitin sulfate. Hyaluronan (800–1,200 kDa; US Biological, 150 intrinsic viscosity units from rooster comb) or chondroitin sulfate (US Biological, type C from shark cartilage) was administered (80 μg each) by two protocols: 1) GAGs were administered 5 min before and at 5-min intervals after vascular obstruction was released. 2) Once the damage to the Gcx was induced, hyaluronan or chondroitin sulfate were injected intravenously at 15, 20, and 25 min following reperfusion.

Reagents and chemicals. Chemicals and reagents were purchased from Sigma, unless otherwise noted. Intravenously and intraperitoneally administered reagents were diluted in sterile physiological saline solution.

Statistical analyses. Experimental and control groups were selected randomly, and each group consisted of 6 mice. All data are expressed as means ± SE. Data were compared using ANOVA and Student's t-tests. Differences were accepted as statistically significant when P < 0.05.

RESULTS

In our model, ischemia was followed during reperfusion by a major increase in arteriolar diameter and capillary flow velocity, but the diameters of capillaries and postcapillary venules were not modified (5.01 ± 0.1 and 6.33 ± 0.2 μm vs. 5.03 ± 0.2 and 6.42 ± 0.3 μm, respectively), findings that are consistent with previous results (48). Over the 20-min baseline period, the average widths of the exclusion zone for Dex70-FITC in capillaries and in postcapillary venules were 0.51 ± 0.01 μm and 0.58 ± 0.02 μm, respectively (Fig. 1A, closed squares and Fig. 2A, shaded bar, baselines). The exclusion...
zones for CMADR-70 were 0.48 ± 0.04 and 0.64 ± 0.12 μm (Fig. 1, open circles and Fig. 2, open bar, baselines) and for Dex 580-FITC were 0.50 ± 0.02 and 0.56 ± 0.03 μm (Fig. 1B, closed triangles and Fig. 2B, hatched bar, baseline). Isoelectric points of each dye were calculated by IEF, Dex70-FITC pI = ~6.75, Dex580-FITC pI = ~6.65, CMADR70 pI = ~6.7, and BSA-FITC pI = ~6.8.

I/R-I caused a rapid, sustained, and statistically significant decrease in the thickness of the Dex70-FITC-exclusion zone in capillaries to 0.26 ± 0.05 μm, ~44% of baseline values (Figs. 1A and 3B), and in postcapillary venules to 0.28 ± 0.08 μm, ~48% of baseline values (Figs. 2A and 4A). I/R-I induced a similar decrease in the CMADR-70 exclusion zone thickness to 0.24 ± 0.07 μm, ~42% of baseline values; and to 0.29 ± 0.06 μm, ~40% of baseline values, respectively (Figs. 1 and 2). In contrast, I/R-I had no effect in the thickness of the Dex 580-FITC exclusion zone either in capillaries or in postcapillary venules (Fig. 1B, closed triangles, and 2B, open bars). For further analysis, we pooled the data from the baseline and the 45-min reperfusion period to make comparisons of the experimental groups.

The RBC-exclusion zone was consistently larger than the Dex70-FITC-exclusion zone in both capillaries (0.86 ± 0.08 vs. 0.51 ± 0.01 μm, P < 0.001) and postcapillary venules (0.91 ± 0.05 vs. 0.58 ± 0.02 μm, P < 0.001), and as shown in Fig. 4, B and C, the RBC-exclusion zone was not significantly affected by I/R in either capillaries or postcapillary venules.

Effects of SOD-Cat. The protective effects of SOD-Cat against I/R-I were evident and statistically significant in both capillaries and postcapillary venules (Fig. 3B, closed squares and Fig. 4A, respectively). The RBC-exclusion zone was not modified by the SOD-Cat treatment in either capillaries or postcapillary venules (Fig. 4, B and C, respectively). SOD-Cat

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**Fig. 1.** A: permeability of glycocalyx is rapidly modified by ischemia-reperfusion (I/R) injury in cremaster capillaries. Baseline values of the Dextran 70 (Dex70)-FITC-exclusion zone decreased significantly following I/R injury (■). Rhodamine-labeled anionic dextran-exclusion zone was also modified after I/R injury as much as Dex70-FITC [carboxymethylamino dextran-rhodamine (CMADR70), ◊]. B: Dextran 580 (Dex 580)-FITC-exclusion zone baseline values were not significantly different from those of CMDAR70 or Dex70-FITC; however, I/R had no effect in the exclusion zone of this large anionic dextran (▲).

**Fig. 2.** A: Dex70-FITC-exclusion zone in postcapillary venules is significantly modified by I/R injury. Also, I/R induced a reduction in the CMDAR70 exclusion zone. B: Dex 580-FITC exclusion zone in postcapillary venules had a similar baseline value as the CMADR70 when perfused at the same time; I/R had no effect on the exclusion zone of this large anionic dextran but significantly reduced the exclusion zone of CMADR70. *Significantly different from baseline values, P < 0.05.
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Fig. 3. A: glycocalyx is rapidly modified by I/R injury in cremaster capillaries. Baseline values of the Dex70-FITC-exclusion zone decreased significantly following I/R injury (○). Dex70-FITC-exclusion zone values were not modified by sham treatment (●) or by the presence of SOD-catalase (Cat) alone experiments (■). B: reactive oxygen species (ROS) scavengers prevent damage of glycocalyx induced by I/R injury in cremaster capillaries. Decrease in Dex70-FITC-exclusion zone induced by I/R injury (○) is prevented by the presence of SOD-Cat maintaining such values at baseline level (■).

Effects of heparin on I/R-I. Intravenous administration of a nonhemorrhagic dose of heparin before reperfusion prevented the deleterious effects of the I/R-I on the Gcx properties studied in our model (data not shown). Figure 5 shows that allopurinol prevented concentration dependently, the deleterious effect of I/R-I on the Dex70-FITC-exclusion zone in capillaries and postcapillary venules, as an indication that XOR is the source of the ROS that mediate the I/R-I effect. The presence of 250 μmol/l allopurinol or its vehicle did not modify the Dex70-FITC-exclusion zone in sham experiments (data not shown). Also, none of the concentrations of allopurinol tested modified the RBC-exclusion zone either in capillaries or in postcapillary venules (data not shown).

Effects of circulating sulfated-GAGs on effects of I/R-I. Chondroitin sulfate infusion did not significantly modify the deleterious effect of I/R-I on either Dex70-FITC-exclusion of capillaries or postcapillary venules (Fig. 6, A and B, respectively). Heparin itself did not modify the RBC or Dex70-FITC-exclusion zones in either capillaries or postcapillary venules in sham experiments (data not shown).

Exogenous hyaluronan reduces and repairs the effects of I/R-I. We administrated high-molecular-weight hyaluronan (800–1,200 kDa) by two protocols, as shown in Fig. 8A. Infusion of hyaluronan before, at, and in the first 5 min of the reperfusion partially prevented the decrease of the Dex70-FITC-exclusion zone induced by I/R-I in capillaries (−62 ± 15% of baseline values, Fig. 8C, closed inverted triangles).

Hyaluronan injected intravenously 15 min after the start of reperfusion (Fig. 8A, open triangles) restored the I/R-induced decrease of the Dex70-FITC-exclusion zone in capillaries (0.54 ± 0.05 μm, ~105 ± 15% of baseline values, Fig. 8C, open triangles). In the absence of I/R-I, the Dex70-FITC-exclusion zone was not significantly modified by the intravenous infusion of high-molecular-weight hyaluronan (Fig. 8B, open squares). Hyaluronan infusion was also effective in reversing (or preventing) the effects of I/R-I on the Dex70-FITC-exclusion zone in postcapillary venules (Fig. 9A). It should be noted that hyaluronan was as effective as SOD-Cat pretreatment in maintaining the Dex70-FITC-exclusion zone levels at baseline values (0.54 ± 0.05 μm, ~105 ± 15% and 0.56 ± 0.03 μm, ~98 ± 9%, respectively). RBC-exclusion zones in mouse cremaster capillaries and postcapillary venules were not modified by the high-molecular-weight hyaluronan (data not shown).

DISCUSSION

Our results show that the Gcx and its constituents are positioned in such a way as to make them both a protective component of the endothelial cell surface and a focal point for microvascular damage in I/R-I. The Gcx is rapidly modified by I/R-I, and the modification appears to be rather selective, with a reduction in the Dex70 exclusion zone without significant effect on the exclusion zone of a larger dextran, Dex 580-FITC, or the intraluminal RBC spacing.

Our data have some implications regarding the processes by which free radicals might be generated and their relation to the constituents of the Gcx. The alteration in the Gcx that we observe appeared to be mediated by the production of ROS as evidenced by the highly protective effect of SOD-Cat. Furthermore, it appears that ROS are enzymatically generated in large part by XOR because its concentration-dependent inhibition by allopurinol greatly reduced the effect of I/R-I on the Gcx. In our model, NADPH oxidase did not appear to contribute to the ROS that damaged the Gcx as shown by the failure of DPI to protect the Gcx against the I/R-I. Based on the protective effect of exogenous heparin and the effect of allopurinol, we propose that the elements that mediate I/R-I (including XOR) are associated with the Gcx via their HBDs. The protective effect
appears to be relatively selective for heparin, because chondroitin sulfate, another sulfate-GAG, did not modify the effects of I/R-I.

I/R-I effects are partially prevented or fully reversed by the intravenous injection of high-molecular-weight exogenous hyaluronan in the mouse cremaster in vivo preparation. Hyaluronan might protect the Gcx because of its antioxidant properties, but the ability of exogenous hyaluronan to rapidly reverse the damage to the Gcx suggests that hyaluronan may actually be able to reconstitute the Gcx, which could have been degraded during the reperfusion.

We estimated the dimensions of the luminal endothelial Gcx with a differential measurement using brightfield and fluorescent microscopy. These measurements allow us to distinguish two regions within the microvessels: one that excludes plasma macromolecules and another that restricts access of RBC. This methodology has been used in our laboratory and by others to show that the Gcx in vivo is target of several stimuli through different mechanisms (25, 47, 48, 63). The exclusion zones appear to define a region of limited access to the endothelial cell membrane, and we assume that the exclusion zones might define the actual in vivo extent of luminal Gcx, i.e., the point of contact between the stationary surface of the endothelium and circulating plasma macromolecules and/or RBCs. It is noteworthy that this exclusion is not simply the result of steric hindrance to the passage of macromolecules but appears to depend on other characteristics of the molecules as well (64, 65). We envision that the Gcx is a selective, complex, and physiologically active structure. Modification of the exclusion zones for either anionic fluorescent dextrans or the RBC by several different stimuli suggests that there is a complex pattern of reorganization of the Gcx following ischemia, which may have differential effects on patterns of dye exclusion and RBC flow patterns (24, 25, 47). In the present investigation, and as previously shown by Platts et al. (48), neither the I/R-I nor any of the treatments used significantly modified the RBC-exclusion zone observed in the cremaster microvessels, whereas I/R-I decreased Dex70-FITC exclusion. Here we show that the exclusion zone for a larger molecular weight dextran Dex 580-FITC is not altered in I/R-I. In contrast, tumor necrosis factor-α reduced both Dex70-FITC, Dex 580-FITC, and the red blood cell spacing (25), suggesting that different structures that comprise the Gcx may be responsible for the interaction with Dex70-FITC, Dex 580-FITC, and RBC.
together, the data suggest that more extensive functional, biochemical, and physicochemical investigations are needed to clearly identify and understand how and which components of the Gcx are responsible for the properties of the full Gcx.

It is well established that one of the early manifestations of the I/R-I is damage to the endothelial cells (21), and in particular, processes associated with the Gcx are often reported to be altered, including cellular swelling (12, 62), macromolecular leakage, and platelet and leukocyte adhesion (40). Figures 3B and 4A show data that argue strongly that, in our model, the protection offered by SOD-Cat-related ROS production play an important role in the I/R injury-induced damage to the Gcx, and these results agree with previous reports that ROS produced by I/R-I are scavenged by SOD-Cat (8, 16, 42). There are other indications that ROS affects the Gcx. The presence of oxidized LDL disrupts the Gcx and increases platelet and leukocyte adhesion (10, 63) and increased capillary tube hematocrit (9). In addition, prolonged epifluorescent light exposure in hamster cremaster microvessels causes a ROS-mediated decrease in the Dex70-FITC exclusion and RBC-exclusion zones (65). In our model, neither the production nor the direct effect of ROS could be solely responsible for the resultant and selective responses in the Gcx modification induced by the I/R-I, which increased only the anionic 70-kDa dextran entry. This difference could be explained by the diverse sources of ROS, dissimilar amount of ROS produced, a gradient sensitivity of the Gcx to ROS, or it could be to the fact that ROS do not have a direct effect on the Gcx (such as...
hydrolyzing GAGs, hydrolyzing proteins that stabilized or anchor those GAGs) but that the ROS may be activating other processes that subsequently would modify the GAGs. In other words, ROS might be part of a more complex signaling process that initiates intracellular signaling events that result in modification of a specific property of the Gcx in cremaster microvessels in response to I/R-I.

We showed previously that adenosine plays an important role in the modification of the Gcx (48). In low concentrations, it can activate an A2A receptor, which protects against I/R-induced damage (48). At higher concentrations, adenosine induced a rapid and sustained decrease of the Dex70-FITC-exclusion zone (47), which may indicate that XOR is involved as a source of ROS. Several enzymatic sources of ROS in mammalian cells have been described, and the most studied in cardiovascular system are NADPH oxidase, XOR, and nitric oxide synthase (6, 34). I/R-I damage has been correlated to enzymatic production of ROS by NADPH oxidase (3, 15, 26, 34, 46, 59, 67), but in our experiments, DPI, an irreversible flavoprotein inhibitor of NADPH oxidase (44), had no significant effect in the damage of the Gcx induced by I/R.

XOR is a luminal, constitutive endothelial enzyme of two isoforms that participate in the metabolism of adenosine by catalyzing hypoxanthine and xanthine into uric acid. Under
normal conditions, NAD$^+$ is used as an electron acceptor in this metabolic reaction (see reviews in Refs. 5 and 36). During ischemia the purine catabolites hypoxanthine and xanthine accumulate, and with reperfusion, as oxygen levels increase, XOR shifts to O$_2$ as an electron acceptor to metabolize the purine catabolites and produce ROS (5, 18, 36). Protective effects of allopurinol in I/R-I have also been reported in other models (19, 30). Thus in this model the major source of ROS would appear to be XOR, but in other models, other enzymes are likely involved (6, 16, 34).

SOD (2) and XOR (1) both possess HBDs, and the in situ activity of the two enzymes can be modifiable by circulating exogenous heparin (1, 2), which appears to displace XOR and SOD, or other molecules with the HBD, from endothelial cell surface. In the present experiments, intravenous administration of exogenous heparin inhibited the modification in the Dex70-FITC-exclusion zone in capillaries and postcapillary venules, suggesting that molecules involved in inducing injury following I/R might be bound to the Gcx through a HBD. If XOR is bound to the Gcx over the endothelial cell surface of the microvessels, the ROS generated from its activity would be produced in immediate proximity to the endothelial cell membrane, and extracellular SOD would transform ROS into hydrogen peroxide, which might either enhance the deleterious effects induced by ROS or be degraded by circulating catalase. This process would thus be in a strategic position to produce damage or to initiate signaling processes that lead to the reorganization of the Gcx, either directly or through another system, such as recruiting leukocytes or mast cells (18, 33, 36). However, heparin effects might be more complex. Interaction of heparin with the endothelial cell may be complex as reported by Jaques and Hiebert (31) in 1989. Effects include activation of antithrombin-III, release of enzymes and chemokines (49), and differential binding and uptake of exogenous heparin, preferentially to larger venules and hepatic sinusoidal capillaries (31). It is noteworthy that in 2003 Constantinescu et al. (10) showed that after the perfusion of oxidized LDL, fluorescent heparin bound to the endothelial cell surface and decreased rolling and adhesion of leukocytes in venules larger than 20 μm but no significant binding to capillaries. Further evaluation of the interaction and heterogeneous effects of heparin in the Gcx are needed.

Hyaluronan is a large, negatively charged polysaccharide (60) of which its structural properties are compatible with it functioning as an important homeostatic component of the Gcx. It has been proposed that the integrity of the hyaluronan on the endothelial cell surface is required to protect the myocardium against edema (62) and that hyaluronan at the endothelial cell surface might modulate the interaction between leukocyte adherence molecules and the leukocyte membrane (32, 37). The latter hypothesis is supported by the data of Henry et al. (24), who showed that hyaluronidase treatment of cremaster microvessels decreased the Dex70-FITC-exclusion zone and that the hyaluronidase-treated Gcx could be restored with the infusion of a mixture of hyaluronan and chondroitin sulfate. Our results show that intravenous administration of hyaluronan alone (at the early stages of reperfusion damage; 5, 0, 5 min after reperfusion) was able to significantly prevent the decrease of the Dex70-FITC-exclusion zone in capillaries and postcapillary venules in response to I/R-I (Figs. 8 C and 9 A). Moreover, intravenous administration of exogenous hyaluronan alone was also effective in restoring the restricted entry of Dex70-FITC into the Gcx at later stages of the reperfusion damage (Fig. 8 C). It is noteworthy that it was previously reported that restoration of the Gcx with exogenous hyaluronan required simultaneous administration of chondroitin sulfate (24, 53), whereas in our model this was not necessary. This difference could be explained by the fact that in the previous experiments, the disruption of the Gcx was caused by administration of exogenous hyaluronidase, which degrades both hyaluronan and chondroitin sulfate, whereas in the present experiments, the spontaneous processes induced by I/R may be more selective and cause degradation of hyaluronan alone.
Hyaluronan has also been reported to be involved in several pathophysiological models, where interstitial tissue and circulating levels of hyaluronan are increased in response to acute stresses (11, 66), such as tumor necrosis factor-α (45, 69).

However, understanding how the hyaluronan participates in the overall response to ischemia remains to be determined. Hyaluronan might be degraded by ROS produced by XOR (39). Other processes may be involved as well, because the mechanisms of turnover of hyaluronan in situ are not yet clearly understood (60).

Modification of the Gcx in I/R-I may involve more complex regulatory mechanisms as well. Enzymes derived from polymorphonuclear leukocytes that are recruited in response to I/R-I may be also be important (20), but to date, polymorphonuclear leukocytes have not been demonstrated to express hyaluronidase activity (17). Also, an increase in the activity of hyaluronidases rather than a change in amount could also be responsible for hyaluronan degradation during I/R, but mechanisms of activation and regulation of these enzymes are not well understood though they are thought to be of critical importance in some pathophysiological states (11).

Recent evidence indicates that oxidative stress might activate several enzymes (61), and ROS produced by XOR in response to I/R-I could be regulating a number of signaling pathways (61). Therefore, it is important to evaluate the participation of the hyaluronan and hyaluronidases in the in vivo I/R-I model.

Understanding the mechanisms involved in the regulation or the modification of the Gcx in response to pathophysiological stimuli will be a very complicated task. The present work contributes by showing that I/R-I induces an alteration in the interaction of the Gcx with the Dex70-FITC, as demonstrated by the reduction in the exclusion zone of this macromolecule, but without effect in the exclusion of larger macromolecules, Dex 580-FITC, and RBC. This reduction is mediated by the production of ROS generated by XOR, an enzyme that is bound to the Gcx through its HBD. This effect could be dependent on the reorganization or loss of Gcx hyaluronan, because the administration of exogenous hyaluronan is able to prevent and reverse the I/R-I-induced effects. As this study demonstrates, hyaluronan could be a key element in the response of I/R-I and that the close interaction between GAGs and mediators of the signaling pathway is critical in the pathophysiologic of the Gcx in vivo.

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