Rapid modification of the glycocalyx caused by ischemia-reperfusion is inhibited by adenosine A2A receptor activation

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Platts, Steven H., Joel Linden, and Brian R. Duling. Rapid modification of the glycocalyx caused by ischemia-reperfusion is inhibited by adenosine A2A receptor activation. Am J Physiol Heart Circ Physiol 284: H2360–H2367, 2003. First published January 30, 2003; 10.1152/ajpheart.00899.2002.—Ischemia-reperfusion (I/R) has been shown to cause microvascular dysfunction and to alter the appearance of the glycocalyx in electron micrographs. We hypothesized that I/R injury might alter the structure and/or permeability of the glycocalyx. Prior work had shown a role for adenosine in protection from I/R injury, and, therefore, we also explored the idea that activation of the adenosine A2A receptor would attenuate I/R glycocalyx injury. Here, we report that I/R causes a rapid and dramatic decrease in the ability of the glycocalyx to exclude FITC-Dextran 70 (Dex70). Over a reperfusion period of 45 min, the glycocalyx dye exclusion zone for Dex70 decreased by one-half in capillaries and post-capillary venules, whereas the red blood cell exclusion zone was very slightly reduced in capillaries only. Pretreatment with the A2A agonist ATL-146e significantly inhibited the changes in both vessel types. The modifications of the glycocalyx appear to be an early step in the inflammatory cascade typically associated with reperfusion injury, and adenosine A2A receptor activation may play a role in protection from this injury.

endothelium; inflammation; permeability; capillary; mast cell

RECENT WORK HAS SHOWN that the true interface between the endothelium and flowing blood is the endothelial glycocalyx rather than the endothelial plasma membrane and that the glycocalyx is both larger and more complex than previously thought. It is becoming apparent that the luminal glycocalyx is typical of other extracellular matrixes, being composed of a combination of glycosaminoglycans, proteoglycans, and glycoproteins. These molecules arise from a poorly characterized combination of endothelium-derived macromolecules and adsorption of various circulating plasma macromolecules (21, 29, 43, 46). This laboratory has previously shown that the matrix of the glycocalyx excludes anionic macromolecules as small as 70-kDa dextran (Dex70) (29, 49, 50) and that it can be as thick as 0.5 μm [rather than the <100-Å value typically seen on electron micrographs (3)]. The glycocalyx is both malleable and dynamic. Permeability, thickness, and uniformity of the glycocalyx can be modified by relatively selective treatment with enzymes such as heparinase (21, 44), hyaluronidase (29), or pronase (2). The glycocalyx also responds to various inflammatory stimuli, including oxidized low-density lipoproteins (13, 48) and TNF-α (30). Moreover, electron microscopy has shown that thickness and uniformity are reduced after ischemia-reperfusion (I/R) (5, 18, 28).

Much remains unclear about the mechanisms involved in I/R injury, but a commonality among proposed mechanisms of I/R injury is microvascular endothelial cell damage and dysfunction associated with increased vascular permeability (8). Possible mediators of this type of injury include free radicals (8, 12, 22, 33), complement activation (12, 33), and mast cell degranulation (34). Leukocyte activation and adhesion have also been reported to contribute to reperfusion injury (8, 12, 33).

A number of previous reports have concluded that I/R can disrupt normal glycocalyx function/structure (5, 19, 28). However, a major limitation of these studies has been the use of electron microscopy in experimental measurements. Preparation of the tissues for electron microscopy is known to cause glycocalyx collapse in many cases and, in so doing, causes substantial underestimation of the size and misinterpretation of the structure of the carbohydrate-rich layer (6, 32, 47). Additionally, electron microscopy studies capture only a single instant in time during or after treatment. Here, we report experiments designed to establish whether I/R produces rapid, dynamic changes in the endothelial cell glycocalyx.

We also chose to explore a possible role for adenosine in the regulation of glycocalyx function during I/R. Adenosine accumulates in ischemic tissues by a variety of mechanisms (4, 38), and, in addition to its role as a vasoactive metabolite, tissue adenosine can act as either a pro- or an anti-inflammatory mediator, depending on concentration and receptor expression (14, 36, 38). All four adenosine receptor subtypes (A1, A2A, A2B, and A3) are reported to be involved in protecting tissue from I/R injury (for reviews, see Refs. 20, 36, and 38), and there is now mounting evidence that activation of...
the A2A receptor can significantly inhibit inflammatory responses (37, 42) and I/R injury (40, 41). The complex role played by adenosine may reflect both concentration-dependent effects and perhaps actions on diverse cell types. We previously showed that activation of the A2A receptor inhibits mast cell degranulation (24), which is a major factor in many inflammatory responses. Others have shown that leukocyte A2A receptors are also involved in anti-inflammatory activity (7, 16, 25). We, therefore, examined the effect of A2A antagonists in an in vivo preparation of an animal with an inguinal hernia. Additionally, we show that adenosine A2A receptor activation provides a substantial protective effect against this form of I/R injury in the mouse cremaster muscle.

METHODS

Animal preparation. All protocols and procedures were approved by the University of Virginia Animal Care and Use Committee and conducted in accordance with the “Guiding Principles in the Care and Use of Laboratory Animals” endorsed by the American Physiological Society. Male C57Bl/6 mice (25–30 g, Hilltop; Scottdale, PA) were anesthetized with intraperitoneal pentobarbital sodium (50 mg/kg body wt in saline). Supplemental anesthesia was given as needed to maintain a surgical plane of anesthesia as determined by monitoring spinal reflexes. All anesthetic injections were made with a dilute solution (6 mg/ml) of pentobarbital, which allows for a more controlled level of anesthesia and maintenance of fluid balance in the animal. Fluid balance was confirmed by measuring systemic hematocrit before experimental manipulation and after the conclusion of each experiment. The left femoral vein was cannulated to allow infusion of FITC-labeled Dex70 solution (Sigma; St. Louis, MO). After the animal was placed on a Plexiglas platter, the right cremaster muscle was prepared for visualization as previously reported (29, 30, 49, 50). Briefly, a midline incision was made in the scrotum, and the testicle was gently separated from the surrounding connective tissue. An incision was then made in the cremaster muscle from the tip of the base, taking care to avoid major blood vessels. The tissue was superperfused at 3 ml/min with a bicarbonate-buffered salt solution composed of (in mM) 131.9 NaCl, 4.6 KCl, 2 CaCl₂, 1.2 MgSO₄, and 20 NaHCO₃. The pH of the solution was maintained between 7.35 and 7.45 by bubbling with 5% CO₂-95% N₂ gas, and the cremaster temperature was maintained at 34°C by warming the superfusion solution. Succinylcholine (10–5 M, Sigma) was added to reduce spontaneous muscle contractions. The cremaster was gently pinned onto a silastic disk mounted on the platter. The tissue was pinned in such a way as to keep the muscle flat and free of slack to allow the proper visualization of the microcirculation. The body temperature of the mice was maintained between 34 and 35°C with a covered copper coil connected to a water bath. During a period of at least 45 min of stabilization, arterioles spontaneously developed tone, and this was taken as a measure of tissue viability. At the end of the stabilization period, a bolus of 0.05 ml Dex70 (20 mg/ml in saline) was given via the femoral vein cannula.

Intravital microscopy. After Dex70 was equilibrated with blood, selected capillaries and postcapillary venules were observed at ×60 (numerical aperture 0.9). A 150-W xenon lamp was used for bright-field and fluorescent measurements. The epi-illuminator was equipped with a 450- to 490-nm excitation filter, a dichroic beam splitter (FT 510), and a barrier filter (LP 520) to permit visualization of FITC-labeled Dex70. Epi-illumination of each vessel was limited to <10 s to minimize light-dye injury to the endothelium. Images of vessels were displayed on an MTI videomonitor and recorded on S-VHS videotapes for subsequent image analysis.

In all groups of animals, global ischemia of the cremaster was induced by cross-clamping the entire cremaster with an atraumatic bulldog clamp (Fine Science Tools; Foster City, CA) as close to the inguinal ligament as possible. Ischemia was maintained for 45 min, at which time the clamp was removed. Ischemia and reperfusion were confirmed by visualization of arteriole networks under low-power magnification. Measurements of the microcirculation were then made (as described in Intravital microscopy) for 45 min. The first group of mice (n = 5) was subjected to the I/R protocol only. A second group (n = 4 mice) was pretreated with the specific adenosine A2A agonist ATL (10 μg/kg ip) (27, 41) 3 min before reperfusion to assess the role of this receptor in I/R injury to the microcirculation. ATL had no effect on systolic blood pressure as measured by tail cuff in anesthetized mice (61.01 ± 2.98 mmHg in ATL vs. 63.67 ± 3.53 mmHg in vehicle control). A third group (n = 4 mice) was pretreated with the A2A antagonist ZM (35 μg/kg) 8 min before reperfusion, followed by ATL 3 min before reperfusion. The final group of mice (n = 3 mice) was a sham control without ischemia, and measurements were made at the appropriate time points as though I/R had been done.

Data and statistical analysis. Data from in vivo experiments were acquired beginning ~5 min after each treatment and continued for up to 45 min postischemia. Recorded video images of the experiments were analyzed using Image-1 software. For each vessel, the anatomic diameter (bright-field microscopy), the width of the Dex70 column (epifluorescence microscopy), and the average diameter of the red blood cell column (bright-field microscopy, 420-nm filter) were measured along a 5.3-μm segment of each vessel using calibrated video calipers. The widths of the red blood cell exclusion zones and Dex70 exclusion zones were calculated as the difference between the anatomic capillary diameter measurements and the width of the red blood cell column divided by two (49). All data are expressed as means ± SE. Mean data were compared using ANOVA and Student’s t-tests. Differences are considered significant at P < 0.05. Unless otherwise noted, all chemicals and reagents were purchased from Sigma.

RESULTS

As estimates of the impact of the glycocalyx on microvessel cross sections, we measured the portion of the lumen that manifested restricted access to Dex70 and the space between the apparent vessel margin and the edge of the red blood cell. Control measurements of all
vessels before ischemia showed a baseline dye exclusion zone of $0.21 \pm 0.01 \, \mu m$ in capillaries ($n = 103$) and $0.23 \pm 0.02 \, \mu m$ in postcapillary venules ($n = 32$). The red blood cell exclusion space was $0.49 \pm 0.01 \, \mu m$ in capillaries and $0.49 \pm 0.02 \, \mu m$ in postcapillary venules.

After I/R, the annulus that excluded Dex70 was rapidly reduced. A significant reduction was observed at 5 min of reperfusion, and, in capillaries, it reached a minimum value of $0.07 \pm 0.02 \, \mu m$ at 20 min (Fig. 2). There was no distinct time-dependent response seen for the red blood cell exclusion measurement. However, when all time points were pooled and compared with pooled control measurements, a small but significant reduction was seen (Fig. 3).

Because the effect of I/R on the glycocalyx is rapid and sustained, time-course responses after I/R (5–45 min) are statistically similar. To compare the responses between groups, the pooled postischemia data for capillaries and postcapillary venules were averaged (Fig. 3). After reperfusion, the portions of the apparent lumen that excluded Dex70 were reduced to $0.10 \pm 0.02 \, \mu m$ in capillaries and to $0.09 \pm 0.02 \, \mu m$ in postcapillary venules. As mentioned above, there was also a 10% decrease in the red blood cell-endothelial cell spacing, to $0.47 \pm 0.01 \, \mu m$ (Fig. 3B). While small, this decrease was highly significant ($P < 0.01$).

In vessels pretreated with the adenosine A2A receptor agonist ATL, the decrease in dye exclusion was delayed, and its magnitude was reduced. In these capillaries, a significant change was seen by 10 min, and the minimum was reached at 25 min (Fig. 2). The maximum response after ATL treatment was 61.4% less than in untreated capillaries. Figure 4 shows that pretreatment with ATL significantly inhibited the decrease in dye exclusion seen after I/R. After ATL, the dye exclusion zone was reduced from $0.22 \pm 0.01 \, \mu m$ to only $0.18 \pm 0.02 \, \mu m$. The response of these vessels was significantly different from both the control and untreated I/R vessels, showing a substantial, but incomplete, inhibition of the I/R injury. The data from postcapillary venules were even more dramatic: ATL completely blocked the change in dye exclusion seen after I/R.

The A2A receptor antagonist ZM was used in an attempt to block the effects of ATL. Vessels pretreated with ZM showed a complete inhibition of the ATL...
effects (Fig. 5). Both dye exclusion (0.2 ± 0.01 μm in control vs. 0.07 ± 0.01 μm with I/R) and red blood cell exclusion (0.48 ± 0.02 μm in control vs. 0.43 ± 0.01 μm with I/R) were similar to control I/R. In fact, the dye exclusion zone in ZM70ATL-treated I/R capillaries was smaller than that with I/R alone (0.10 ± 0.001 μm in I/R alone vs. 0.07 ± 0.008 μm in I/R with ZM and ATL together).

DISCUSSION

**General.** I/R injury associated with coronary bypass, organ transplantation, thrombolytic therapy, and other procedures produces a pronounced inflammatory response with potentially severe clinical consequences (8, 12, 22). It is clear that one of the primary manifestations of reperfusion injury is microvascular dysfunction, which is centered largely on the endothelial cell (8, 12). Microvascular dysfunction includes increased permeability, cell swelling, increased expression of endothelial adhesion molecules, increased leukocyte adhesion (1, 8, 12), and disruption of the glycocalyx (5, 17, 28). While the molecular causes of the dysfunction are not clear, previous work in this laboratory has shown that the glycocalyx is a dynamic structure that can respond rapidly to a number of stimuli, including production of reactive oxygen species (49) and TNF-α (30). Both of these stimuli are known to be involved in inflammatory responses and have been implicated in mediating I/R injury. There is evidence linking disruption of the glycocalyx to leukocyte adhesion (35, 39), platelet adhesion (48), and increased microvascular permeability (2, 31), all of which are components of I/R injury. Thus we hypothesized that various aspects of glycocalyx function would be modified after I/R, which is, at least partially, associated with a well-documented inflammatory component (8, 26, 33).

**Other glycocalyx I/R studies.** Several studies using electron microscopy have shown that I/R injury causes significant damage to the endothelial glycocalyx (5, 17, 28). One of these studies found that the glycocalyx was entirely removed from the endothelium after 1 h of ischemia and 1 h of reperfusion (28) of feline coronary capillaries. Two other studies, however, showed that the glycocalyx of isolated perfused hearts became disrupted, presenting a “floculent” appearance after experimental I/R (5, 17). Our in vivo data from mice corroborate and extend these studies. Tissue preparation for electron microscopy causes the glycocalyx to collapse (6, 32), and it is not known which components of the glycocalyx remain and which are removed or altered by fixation and dehydration. Thus it is difficult to correlate the anatomic appearance of the glycocalyx in electron micrographs with our in vivo measurements using Dex70 or red blood cell exclusion. Taken together, however, we believe that these studies show the importance of the glycocalyx in I/R injury and lend support to our hypothesis that the glycocalyx is an early target of reperfusion injury.

**Fig. 3.** Average responses to I/R for capillaries and postcapillary venules. A: Dex70 exclusion from the glycocalyx before and after I/R. Red blood cell (RBC) exclusion properties are shown in B. Bars represent means ± SE. The number of vessels observed is indicated in each bar. *P < 0.05.

**Fig. 4.** ATL significantly inhibits I/R damage to the glycocalyx. A: effects of ATL on dye exclusion from the glycocalyx in capillaries and postcapillary venules. The inhibition is significant in both vessel groups but not complete in capillaries. Complete inhibition of changes in RBC exclusion characteristics are seen in B. Bars represent means ± SE. The number of vessels observed is indicated in each bar. *Significantly different from control, P < 0.05; §significantly different from I/R treatment, P < 0.05.
Adenosine A2A receptor. Adenosine accumulates in tissues during ischemia, and there is substantial evidence that it plays a complex role in responses to ischemia, functioning as a pro- or an anti-inflammatory agent (14, 15, 36). The complexity of the adenosine response is likely a reflection of the fact that four different receptor subtypes are expressed (A1, A2A, A2B, and A3) in varying degrees on several different vascular effector cells, including mast cells, leukocytes, and the endothelium. This diversity creates a complex system in which adenosine may cause varying effects depending on concentration, receptor subtype expression, and the cell types present (23, 36, 45).

Much effort has been expended elucidating the role of the adenosine A1 and possibly A3 receptors in preconditioning (20, 38). Recently, evidence has begun to accumulate pointing to a key role for the A2A receptor as a source of tissue protection in a variety of I/R models (11, 41, 42). ATL is a specific agonist for the adenosine A2A receptor and has been shown to provide protection from reperfusion injury (10, 41).

We pretreated animals with ATL shortly before (3 min) reperfusion and found that activation of the adenosine A2A receptors significantly inhibited reperfusion injury to the glycocalyx as shown by the marked decrease in glycocalyx porosity to Dex70 (Figs. 2 and 4). Because the protective effect of ATL has also been shown to be completely blocked by ZM, a selective A2A antagonist (41, 42), we performed a series of experiments using both molecules. The ZM compound was injected 5 min before ATL and 8 min before reperfusion. The ZM compound successfully blocked the protection seen with ATL alone. This is strong evidence that the adenosine protective effect on the glycocalyx is mediated via the A2A receptor, as hypothesized.

The cell type that is primarily responsible for the protection offered by ATL remains to be demonstrated. The A2A receptor is known to be expressed on the endothelium, mast cells, leukocytes, macrophages, and platelets (8, 12, 33, 36). Each of these cells has been implicated in mediating some aspect of I/R injury, and each could affect the glycocalyx by release of reactive oxygen species, proteases, or cytokines. Future experiments will be conducted to determine which cell type has the greatest effect on the glycocalyx after I/R in our model.

Model. To assess the function of the glycocalyx, we measured two distinct, though perhaps related, parameters. The first is the ability of the glycocalyx to exclude Dex70 from a region immediately adjacent to the luminal endothelial membrane (29, 30, 49, 50). We used Fig. 6. Model of capillary glycocalyx under control conditions (left) and after I/R (right). The capillary glycocalyx is shown as simplified line drawings, where the white mottled background represents the dye exclusion zone seen measured in our experiments. The glycocalyx is composed of proteoglycans, hyaluronan, and glycoproteins from various sources. Right: one interpretation of what happens to the matrix after I/R. Notice that some of the hyaluronan cross-linking molecules are gone and that the dye exclusion layer is reduced. Some molecules remain intact to maintain a large fraction of the RBC exclusion zone. [Adapted from Ref. 29.]
this measurement to gauge a combination of the size and permeability of the glycocalyx. As the glycocalyx is reduced or it becomes less able to exclude the dye, the apparent thickness measured with Dex70 decreases. The second measurement is the red blood cell exclusion zone. This is a more complex measurement that includes the full thickness of the glycocalyx, the so-called plasma slip layer (9, 30, 49), as well as any other surface phenomena that might be associated with the endothelium. It is currently unknown which measurement more closely approximates the true thickness of the glycocalyx. It is our opinion, however, that the dye measurement may be an underestimate of the true glycocalyx dimension and more accurately represents movement of macromolecules into this matrix. It is also possible that the matrix extends beyond the red blood cell and is constantly compressed by flowing red blood cells. At this time, we cannot state the absolute glycocalyx thickness, only a relative thickness that is reproducibly regulated by various stimuli. We included both measurements because of the potential for increased understanding that comparisons of the two measurements may bring.

Our present data, as well as previous work (29, 30, 49), show that the red blood cell spacing and dye exclusion can be affected to different degrees by different stimuli. Henry and Duling (29) demonstrated that enzymatic degradation of hyaluronan increased the movement of Dex70 into the glycocalyx, without significantly changing the red blood cell exclusion properties. Additionally, we more recently found that TNF-α modified both the Dex70 and red blood cell exclusion properties. In that study (29), dye exclusion decreased ~75%, whereas red blood cell exclusion decreased by only 14%. Our current data show that I/R causes a rapid decrease in glycocalyx exclusion of Dex70, a molecule we have used extensively to assess glycocalyx function (29, 30, 49, 50). This decrease was seen in both capillaries and postcapillary venules. At the same time, we also saw a smaller but significant decrease in the ability of the glycocalyx to exclude red blood cells. The magnitude of the change in dye exclusion is roughly twice that of the change in red blood cell exclusion. These data show similar patterns as found in earlier work, leading to the conclusion that the glycocalyx is a complex matrix that consists of multiple functional elements, each of which may be differentially altered by treatment, leading to differences between the effects on dye and red blood cell exclusion characteristics. Earlier enzymatic work suggests that hyaluronic acid is important for regulation of macromolecule (dye) exclusion (29), whereas heparan sulfate is required for overall glycocalyx integrity (red blood cell exclusion) (21, 44).

It appears that I/R leads to a “loosening” of the glycocalyx matrix, possibly by disrupting the interactions between proteoglycans and hyaluronic acid (Fig. 6). This loosening seems to increase the porosity of the matrix, allowing the FITC-Dex70 dye to penetrate closer to the endothelial membrane. The effect of I/R on red blood cell spacing is much less, however, and this change might also be the result of a looser matrix, which might compress more easily in response to the shear forces exerted by flowing blood. This would cause a decrease in the measured distance between the red blood cell and the capillary wall. The degree of matrix damage would determine the exclusion characteristics of the matrix, with lesser damage affecting only macromolecule penetration and more severe damage affecting both macromolecules and red blood cells. To our knowledge, there are no instances when the red blood cell exclusion properties are modified without a concurrent change in macromolecule exclusion.

There may be an additional component to the structure of the glycocalyx. The reduction in the size of the space from which dye is excluded plateaus around 0.1 μm from the endothelial surface (Fig. 2). This may indicate a third compartment of the glycocalyx that is highly resistant to degradation and forms the “last line of defense” for the endothelium. Figures 2–4 show that there is a space adjacent to the endothelial cell surface that, although reduced, is not eliminated by I/R.

Although the movement of the dye front seems arrested at a distance of ~0.1 μm from the cell surface, it is clear that dye does leave the vasculature because we can observe its accumulation in the muscle tissue. This accumulation is increased after ischemia. We also noted a remarkable accumulation of dye in perivascular phagocytic cells, which we have yet to identify, that seem to have the characteristics of dendritic cells. Although we have yet to make simultaneous measurements of the glycocalyx and vascular permeability in this model, other investigators have shown that the glycocalyx contributes significantly to the permeability barrier (2, 31).

In summary, we have shown that the endothelial glycocalyx of capillaries and postcapillary venules is rapidly modified after I/R. We also found that this modification is significantly inhibited by pretreatment with an agonist to the adenosine A2A receptor, ATL, and that the ATL effect can be reversed with an A2A antagonist (ZM). Additionally, our data are consistent with the idea that modification of the glycocalyx is an early step in the cascade of events leading to I/R injury, including increased adhesion of leukocytes and platelets and increased microvascular permeability. This leads to the possibility that modification of the glycocalyx is a primary component of early I/R injury and suggests a possible new therapeutic target in the prevention and/or treatment of I/R injury.

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