Shedding of the coronary endothelial glycocalyx: effects of hypoxia/reoxygenation vs ischaemia/reperfusion

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Editor’s key points

- The gel-like glycocalyx protects the lumen of the endothelium.
- Damage to the glycocalyx is important in critical illness.
- This study measured glycocalyx damage after ischaemia or hypoxia in guinea pig hearts.
- Both ischaemia and hypoxia caused damage and mast-cell degranulation may contribute.

Background. Vascular endothelium is covered by a glycocalyx. Damage to the glycocalyx after systemic inflammation or ischaemia/reperfusion contributes to increased vascular permeability and leucocyte adhesion. The underlying mechanisms leading to ischaemia/reperfusion-induced glycocalyx shedding are incompletely understood, in terms of lack of oxygen, absence of flow, or return of oxygen.

Methods. Isolated guinea pig hearts perfused with Krebs–Henseleit buffer at 37°C underwent 20 min of either stopped-flow ischaemia or hypoxic perfusion with subsequent reperfusion/reoxygenation (n=6 each). Hearts perfused with normoxic buffer served as time controls. Epicardial transudate was collected to assess coronary net fluid filtration, colloid extravasation, and histamine release by mast cells. Syndecan-1 and heparan sulphate were measured in coronary effluent, together with lactate, purines, and the release of mast-cell tryptase β. Additional hearts were perfusion-fixed to visualize the glycocalyx.

Results. Both ischaemia and hypoxia with reperfusion/reoxygenation resulted in significant increases in net fluid filtration (P<0.05) and release of syndecan-1 and heparan sulphate in coronary effluent. These effects were already seen with the onset of hypoxic perfusion. Histamine was released during hypoxia and reoxygenation and also reperfusion, as was tryptase β, and high concentrations of adenosine (>1 μmol litre⁻¹, hypoxia group) and inosine (>7 μmol litre⁻¹, ischaemia group) were measured in effluent (P<0.05). Damage to the coronary glycocalyx was evident upon electron microscopy.

Conclusions. Both ischaemic and hypoxic hypoxia initiate glycocalyx degradation, promoting an increase in permeability. A contributing mechanism could be purine-mediated degranulation of resident mast cells, with liberated tryptase β acting as potential ‘sheddase’.

Keywords: heart, ischaemia; heart-isolated preparation; hypoxia; microcirculation; vascular

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The vascular endothelium is severely affected during critical illness. It is becoming increasingly evident that damage to the glycocalyx, covering the luminal surface of normal endothelium, represents the earliest stage of endothelial perturbation.1 2 This makes the endothelial glycocalyx an interesting target for protective interventions in the critical care setting.1 3 4 Together with the endothelial cells, the glycocalyx builds up a ‘double barrier’ against fluid and protein extravasation, with both components being essential for effective barrier function.5 The glycocalyx consists of core proteoglycans of the syndecan and glypican families, carrying a highly sulphated fringe of glycosaminoglycans (heparan, chondroitin and dermatan sulphates), and receptor-bound hyaluronan. Together with solubilized glycosaminoglycans and plasma proteins, the glycocalyx forms the endothelial surface layer.6 This gel-like structure represents the real physiological principle at the interface between the endothelial cell and the flowing blood.7 Currently, little is known about the mechanisms controlling the balance of synthesis and degradation of the glycocalyx. During pathological conditions, release of proteases, nitric oxide, and reactive oxygen species interfere in this process.8–10

Clinical and experimental observations show that the glycocalyx is severely affected after ischaemia/reperfusion.11–13 The question remains open if low oxygen tension, the absence of flow during ischaemia, or the return of oxygen...
is the main trigger for destruction. After ischaemia, initiation of reperfusion seems to be crucial for the shedding process. In contrast, former morphological studies using electron microscopy have shown disruption and loss of the endothelial cell glycocalyx, termed ‘shedding’, after a period of complete anoxic perfusion without sequential reoxygenation. Here again, it is unresolved whether complete anoxia is necessary, or if hypoxia—the clinically more common state of malperfusion—is sufficient to initiate glycocalyx degradation.

To clarify these issues and to determine possible differences between shedding induced by hypoxic and ischaemic insults, respectively, we used an isolated perfused heart model designed to facilitate detection of the state of the glycocalyx. Particular attention was focused on the links to the functional state of the vascular barrier, metabolic phenomena, mast-cell degranulation, and insulin-induced shedding. Resident mast cells were of interest, because they produce numerous inflammatory mediators, are located perivascularly, and release various proteases upon stimulation. In the human heart, they have recently been found to be the only store of the enzyme heparanase, required to shed heparan sulphate moieties from the glycocalyx. We hypothesized that hypoxic perfusion is sufficient to initiate degradation of the endothelial glycocalyx.

Methods

The investigation conforms to the Guide for the Use of Laboratory Animals published by the National Institute of Health, USA, and was approved by local governmental authorities (No. 209.1/211-2531.3-3/99).

Heart preparation

Guinea pig hearts were isolated and perfused in a modified Langendorff model as previously described. Male guinea pigs, median weight (inter-quartile range (IQR)) 270 (260–300) g, were randomized to different experimental groups by choosing pre-prepared envelopes. After stunning (260–300) g, were randomized to different experimental groups by choosing pre-prepared envelopes. After stunning (260–300) g, were randomized to different experimental groups by choosing pre-prepared envelopes.

Experimental protocols

After preparation, an equilibration interval of 15 min was allowed to establish steady-state conditions in the spontaneously beating hearts. After baseline measurements, hearts were treated according to three different protocols (n=6 hearts each): Group A (control) hearts served as time control, being perfused for 55 min with well-oxygenated (gassed with 95% oxygen) Krebs–Henseleit buffer. In Group B (hypoxia/reoxygenation), perfusion was continued after baseline measurement with an hypoxic (gassed with 21% oxygen) Krebs–Henseleit buffer for 20 min, followed by reoxygenation (oxygen 95%) for another 20 min. In Group C (ischaemia/reperfusion), a period of 20 min stopped-flow ischaemia (37°C) was followed by 20 min of reperfusion (oxygen 95%). After finishing the protocols as described above, concentrated hydroxyethylstarch solution (HES 130/0.4, Fresenius Kabi, Germany) was added at a final concentration of 1% HES to the perfusate in all groups. Perfusion was continued for 20 min to allow determination of colloidal extravasation into the transudate. At the end of this phase, the atria were trimmed off and the ventricles dried at 60°C for 24 h to determine the heart weight independent of oedema.

Calculation of oxygen delivery

Partial pressure of oxygen was determined in a blood gas analyzer at 37°C. Oxygen content of the Krebs–Henseleit buffer was calculated with the Bunsen coefficient (0.023 ml oxygen ml⁻¹ at 760 mm Hg). Oxygen delivery was calculated by multiplying the actual total coronary inflow rate (summed effluent and transudate flow) with the oxygen content in the respective sample.

Release of glycocalyx components, histamine, and tryptase β-like activity

Shedding of soluble glycocalyx components syndecan-1 (CD-138) and heparan sulphate was assessed in coronary effluent during hypoxic perfusion (Group B), reoxygenation (Group B), or reperfusion (Group C) or during corresponding time frames in the control group (Group A). Histamine concentrations were determined in transudate by ELISA (Spibio, Montigny le Bretonneux, France). Tryptase β-like activity in coronary effluent was determined by modifications of described methods. Activity was related to the heart weight and total volume of effluent sampled during the different experimental phases (for details, see Supplementary material).

Determination of HES concentrations and measurement of lactate and purines

Concentrations of HES were quantified in coronary effluent and transudate using a method described previously. In brief, it involves hydrolysis to glucose and subsequent enzymatic/photometric determination of the sugar. Lactate, adenosine, and inosine were determined by high-performance liquid chromatography in samples of coronary effluent.
according to Becker and colleagues19 (for details, see Supplementary material).

Electron and light microscopy
Additional hearts (three per group) were perfusion-fixed with lanthanum/glutaraldehyde mixture according to previously described procedures.12 20 The hearts were fixed after 20 min ischaemia, 20 min of hypoxia, 20 min reperfusion, or 20 min of reoxygenation. Hearts with 55 min of normoxic perfusion served as time controls.

Additional guinea pig heart tissue was fixed for adenosine A3-receptor immunostaining (see Supplementary material).

Statistics
Previous experiments have shown sample sizes of about n=6 to suffice for statistical evaluation of biologically relevant effects. Data were considered to be not normally distributed and are given as median with IQR or full range, unless indicated otherwise. Differences between groups at a single time point were compared by the Kruskal–Wallis one-way ANOVA on ranks (three groups) or a Mann–Whitney rank-sum test (two groups A and B, without ischaemia group C), followed by a Student–Newman–Keuls test, as appropriate. Correlation coefficients between different parameters were calculated. A value of P<0.05 was considered statistically significant (SigmaStat, Systat Software, Richmond, VA, USA).

Results

Coronary flow
The median (IQR) basal effluent flow rate for all hearts was 7.9 (6.5–8.2) ml min⁻¹, there being no statistically significant difference among groups (Table 1). Reactive hyperaemia was only significant within the first minute of reperfusion (data not shown). With ongoing reoxygenation (Group B) or reperfusion (Group C), the coronary effluent flow rate decreased significantly (P<0.05) over time (Table 1).

Oxygen delivery
Oxygen supply at baseline did not differ among groups. With the onset of hypoxia (Group B) or ischaemia (Group C), oxygen delivery was significantly lower than in the control group (Group A). After reperfusion or reoxygenation, there was no significant difference in oxygen supply among the three groups (Table 1).

Fluid filtration
The median (IQR) basal transudate formation was 0.37 (0.31–0.55) ml min⁻¹, with no significant difference among groups. This amounted to about 5.2% of basal coronary venous effluent flow. As actual coronary flow may influence filtration rate, net fluid filtration was expressed as a ratio between actual transudate formation and effluent flow rate (Fig. 1). During hypoxic perfusion, fluid filtration increased significantly (P<0.05) compared with control conditions. During reoxygenation, fluid filtration tended to decrease again, but remained significantly increased compared with control in these hearts. Also, after reperfusion of ischaemic hearts, transudate to effluent ratio increased significantly (P<0.05) vs control (Fig. 1).

HES extravasation
In the control group, a median (IQR) of 6.9 (5.6–7.8)% of applied colloid appeared in transudate after equilibration. Ischaemia/reperfusion increased HES extravasation to 16.6 (8.9–32.5)% (*P<0.05 vs control). After hypoxia/reoxygenation, HES extravasation was 10.7 (8.9–20.2)%, which was not significantly higher.

Measurements of glycocalyx constituents
During 20 min of hypoxic perfusion, summed CD-138 release increased significantly in Group B compared with the well-oxygenated control group A (P<0.05; Table 2). Heparan sulphate concentrations were not significantly higher in Group B during hypoxia. During the 20 min of reoxygenation (Group B) and during reperfusion (Group C), both summed CD-138 and heparan sulphate release values lay significantly lower than Group A (P<0.05).

Table 1 Coronary flow and oxygen delivery. Coronary effluent flow (ml min⁻¹) and oxygen delivery (ml min⁻¹) during baseline, 20 min ischaemia/hypoxia (I/H) and 20 min reperfusion/reoxygenation. Data are given as median (IQR), n=6 per group. Coronary flow: *P<.05 Group B reoxygenation vs basal and hypoxia; #P<.05 Group C reperfusion vs basal and ischaemia. Oxygen delivery; 7P<.05 Group B and Group C vs Group A; 8P<.05 Group C vs Group B

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<th>Basal</th>
<th>I/H</th>
<th>Reperf./Reoxygenation</th>
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<td><strong>Coronary effluent flow (ml min⁻¹)</strong></td>
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<tr>
<td>Group A (control)</td>
<td>8.2 (6.4–9.0)</td>
<td>7.7 (5.5–8.7)</td>
<td>6.3 (5.2–6.6)</td>
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<tr>
<td>Group B (HR)</td>
<td>8.0 (6.8–8.1)</td>
<td>8.4 (4.8–9.2)</td>
<td>6.0 (3.8–7.1)*</td>
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<td>Group C (IR)</td>
<td>7.1 (6.5–8.0)</td>
<td>0.00 (0.0–0.0)</td>
<td>5.8 (5.5–6.2)+</td>
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<tr>
<td><strong>Oxygen delivery (ml min⁻¹)</strong></td>
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<tr>
<td>Group A (control)</td>
<td>0.16 (0.12–0.17)</td>
<td>0.15 (0.10–0.17)</td>
<td>0.13 (0.10–0.13)</td>
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<tr>
<td>Group B (HR)</td>
<td>0.15 (0.13–0.15)</td>
<td>0.03 (0.02–0.04)+</td>
<td>0.13 (0.08–0.15)</td>
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<tr>
<td>Group C (IR)</td>
<td>0.14 (0.12–0.15)</td>
<td>0.00 (0.00–0.00)+,+</td>
<td>0.12 (0.10–0.12)</td>
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higher compared with control conditions \((P<0.05\) vs Group A; Table 2). Release did not differ significantly between Groups B and C during the reperfusion/reoxygenation phase (Table 2).

**Lactate**

Lactate release increased significantly during hypoxic perfusion (Group B) compared with the corresponding control hearts (Group A). With the onset of reoxygenation, lactate values decreased again, but still remained significantly elevated for the first 5 min. After ischaemia (Group C), a singular significant peak in lactate release occurred immediately upon reperfusion (Fig. 2).

**Adenosine and inosine**

Adenosine and inosine concentrations in coronary effluent of the control group (Group A) remained low and unchanged throughout the experimental course (Figs 3 and 4, respectively). During hypoxic perfusion (Group B), adenosine concentration increased rapidly, reaching a peak after 10 min \((P<0.05)\). Then, values started to decrease gradually, but remained significantly elevated compared with the control group until after the first 5 min of reoxygenation. Also after ischaemia (Group C), the adenosine concentration was elevated about five-fold above basal (Fig. 3). Although the increase was significant compared with control, the value attained remained much lower than those seen during hypoxia in Group B \((P<0.05)\; \text{Fig. 3}). A similar time course was noted for inosine (Fig. 4), but the concentrations were about one order of magnitude higher than those of adenosine. Also the increment for inosine observed immediately upon reperfusion in Group C led to levels much higher than in the hypoxic group B \((P<0.05)\).

**Histamine release and tryptase \(\beta\)-like activity**

As listed in Table 3, histamine release was significantly elevated under hypoxic conditions, normalizing during reoxygenation (Group B). Reperfusion after ischaemia was associated with significantly elevated washout of interstitial histamine (Group C; Table 3). Similar results during hypoxia/reoxygenation and reperfusion were seen for tryptase \(\beta\)-like activity in coronary venous effluent (Table 3). This indicates

**Table 2** Soluble markers of glycocalyx shedding. Total syndecan-1 (CD-138) and heparan sulphate release per gram dry heart weight in coronary effluent during 20 min hypoxia, 20 min reperfusion/reoxygenation, or corresponding time (controls). Data are given as median, IQR, and full range \((n=6)\). The dashed line represents the median basal ratio for all hearts. \(*P<0.05\) hypoxia vs control; \({}^{\#}P<0.05\) reoxygenation and reperfusion vs control.

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<tr>
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<th>Ischaemia/hypoxia</th>
<th>Reperfusion/reoxygenation</th>
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<tr>
<td>CD-138 release ((\text{ng} \ \text{g}^{-1}))</td>
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<tr>
<td>Group A (control)</td>
<td>24.7 (19.7–260)</td>
<td>73 (25–171)</td>
</tr>
<tr>
<td>Group B (HR)</td>
<td>982 (824–1092)*</td>
<td>695 (319–1000)*</td>
</tr>
<tr>
<td>Group C (IR)</td>
<td>—</td>
<td>446 (285–588)*</td>
</tr>
<tr>
<td>Heparan sulphate ((\mu\text{g} \ \text{g}^{-1}))</td>
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<td></td>
</tr>
<tr>
<td>Group A (control)</td>
<td>1.7 (1.2–5.6)</td>
<td>0.5 (0.1–0.7)</td>
</tr>
<tr>
<td>Group B (HR)</td>
<td>3.7 (3.0–8.2)*</td>
<td>3.5 (1.7–5.6)*</td>
</tr>
<tr>
<td>Group C (IR)</td>
<td>—</td>
<td>7.5 (3.0–20.7)*</td>
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significant activity of mast-cell proteases, not only in the perivascular interstitium, but also in the endovascular lumen.

Correlation analysis
There was a good correlation between release of histamine and that of tryptase β in our experiments (correlation coefficient 0.59; P<0.05). Correlations between peak purine concentrations and tryptase, and tryptase and soluble glyco- calyx components failed to reach statistical significance (data not shown).

Discussion
The ‘double barrier’ against fluid extravasation composed of endothelial cells and the glyco- calyx is severely affected by ischaemia/reperfusion. Ischaemia alters the endothelial cell shape and therefore modulates the second barrier. In combination with glyco- calyx shedding, one may expect an increase in both hydraulic conductivity and colloid permeability. Conversely, during anoxia, morphometric studies showed that the narrow zones of the capillary interendothe- lial clefts become narrower. Later, ultrastructural examinations showed increased gap formation in endothelial venules during experimental anoxic perfusion. As solely increasing gap width between venular endothelial cells should not increase vascular permeability for colloids, we focused on the endothelial glyco- calyx in the current investigation.
We now demonstrate that glycocalyx shedding is neither dependent on anoxia nor on the combination of the absence of flow and oxygen (ischaemic hypoxia). Instead, hypoxic conditions with preserved flow (hypoxic hypoxia) also suffice to initiate rapid degradation. Increased net fluid filtration was accompanied by a significant increase in the washout of soluble glycocalyx components in the coronary venous effluent during both reoxygenation and reperfusion. Vascular leakage and release of glycocalyx components were apparent already within 5 min of hypoxic perfusion. Thus, return of oxygen is not the initializing culprit. Damage to the glycocalyx could also be visualized by electron microscopy in all hypoxic groups. These quantitative and qualitative results are in accordance with previous, morphological studies, which demonstrated a disruption of rat coronary capillary endothelial glycocalyx after complete anoxic perfusion without subsequent reoxygenation.15

Interestingly, despite a tendency to increase, HES extravasation after hypoxia/reoxygenation did not increase statistically significantly. This may be due to less severe endothelial cell perturbation, or more likely, to the existence of a somewhat more pronounced residual glycocalyx. Addition of a colloid then yields a rudimentary endothelial surface layer allowing better intravascular retention of fluid and colloid. In contrast to the ischaemia/reperfusion setting, continuous flow preserved during hypoxic hypoxia facilitates washout of mediators, proteases, and heparanase, possibly reducing net damage. This interpretation is consistent with investigations showing a more intense effect of enzymatic glycocalyx degradation with exogenously applied heparinase if, additionally, stopped flow had been initiated.5 Rapid removal of damaging agents may also explain the areas of preserved glycocalyx observed by electron microscopy after hypoxia/reoxygenation in selected capillaries (Supplementary Fig. 5). Alternatively, we cannot rule out rapid partial recovery of the glycocalyx, perhaps by externalization from caveolae. These have been found to contain densely packed glycocalyx.23 Considering that days are necessary for total recovery of a destroyed glycocalyx in vivo, full restoration cannot be expected in our isolated heart model.24

There are several mechanisms which may contribute to shedding: increased glycocalyx shedding can be triggered by the atrial natriuretic peptide and inflammatory stimuli like tumour necrosis factor (TNF)-α.25–27 Mechanisms may involve membrane-bound proteases, reactive oxygen radicals, and activated inflammatory cells entering affected tissues during reperfusion, and activation of resident mast cells.1,8,28 Modulation of the capillary endothelial glycocalyx by adenosine has been shown by Platts and Duling: while activation of A2A receptors may protect the glycocalyx from ischaemia/reperfusion-induced modifications, a decrease in exclusion properties of the glycocalyx can be initiated via the low-affinity A3-type receptor.29,30 Also, Brands and colleagues31 showed a significant release of the glycocalyx component hyaluronan after adenosine application in goat hearts. Both hypoxic and ischaemic hypoxia lead to release of adenosine from the heart because of decreased salvage of nucleoside and enhanced catabolism of adenine nucleotides.32 High concentrations of adenosine in the micromolar range could be detected in coronary venous effluent with the onset of hypoxic perfusion in our experimental model, exceeding released concentrations in the ischaemic group after reperfusion by far. In contrast, the concentrations of inosine in coronary effluent were about two orders of magnitude higher than those of adenosine in the ischaemic group after reperfusion, reaching levels of 10 μM. A possible explanation for this observation is further degradation of adenosine, built up during ischaemic stasis, to inosine by adenosine deaminase. Alternatively, intracellular acidosis may activate AMP deaminase, leading to IMP and, by means of 5′-nucleotidase, to inosine.32

A3 activation by high concentrations of adenosine (nearly 1 μM) is known to cause degradation of tissue mast cells, releasing multiple inflammatory mediators.33 The TNF-α, released during myocardial reperfusion, comes from mast cells.34 Activation of tissue mast cells could be demonstrated in our experiments by the significantly correlated release of histamine and tryptase β, both in the hypoxic and ischaemic groups. The adenosine concentrations measured here after ischaemia would not seem to be high enough to cause mast-cell degranulation.33 However, inosine also activates the A3 receptor on mast cells, and concentrations measured during reperfusion in Group C were high.33,35 Therefore, purine signalling is a likely explanation for mast-cell degranulation in our experiments. Pertinently, very recent studies have shown resident mast cells to be the sole storage site of heparanase in the human heart.16 The release of tryptase β-like activity into the coronary system represents another candidate ‘sheddase’.

There was no significant correlation between purine concentration and released tryptase activity, nor between tryptase release and soluble glycocalyx components in the coronary effluent. However, tryptase may not be the sole ‘sheddase’ in our scenario, and in addition, the regional distribution of mast cells and total histamine content has been shown to vary between individual guinea pig hearts.16 A possible role for mast-cell stabilization in protection against ischaemia/reperfusion injury has already been shown in other experimental settings.34,37

The whole organ preparation we used allows the description of net effects involving different vessel types in an intact vascular bed with the myocardial contractions inducing a physiological pulsatile flow in the vasculature. However, local changes in the surface area and differences in diastolic time fractions may influence net-filtration rate. To minimize such confounding factors, which may also affect total coronary flow, we calculated a ratio between transudate formation and effluent flow rate for comparison of net fluid filtration between groups. Hearts were perfused with artificial, plasma-, and cell-free medium, leading to some tissue oedema after 30 min of reperfusion, as noted previously.5 This was evident also in the control group, despite visualization of a seemingly intact glycocalyx. Oedema may
contribute to the decrease in coronary effluent flow over time observed in both interventional groups. As we used a constant perfusion pressure, this is a sign of increased vascular resistance of the whole organ preparation. Our results are in agreement with former studies detecting an increase in vascular resistance after hypoxic perfusion of isolated hearts with cell-free buffer.\textsuperscript{2,3} Despite the ability of the heart to compensate for low oxygen supply by increasing flow through individual areas (dilatation or capillary recruitment), this may not compensate for resistance offered by swollen capillary endothelium after prolonged perfusion. Finally, plasma borne factors of the complement and coagulation systems, pro- and anti-inflammatory enzymes, and granulocytes and platelets are important contributors to ischaemia/reperfusion- and hypoxia/reoxygenation-induced injury. These confounding factors are absent from our blood-free preparation, and further investigations, preferably in \textit{vivo}, are necessary to study other effects on the glycocalyx.

**Conclusion**

We demonstrated that both ischaemia and hypoxia are sufficient to initiate a significant shedding of the endothelial glycocalyx, leading to a profound increase in vascular permeability. The mechanism seems to involve degranulation of resident tissue mast cells, probably triggered by the generation of high concentrations of adenosine and inosine during hypoxia and ischaemia, respectively. Although mast cells are likely to be of great significance, one should not expect them to be the sole initiators of shedding in all scenarios. Vascular permeability remained elevated during ongoing reperfusion and reoxygenation, though showing a tendency towards better recovery in the latter group. However, this distinction might only apply to the short time span of the follow-up in the present model. Further studies are needed to show links between effects of chronic and acute hypoxia on endothelial glycocalyx degradation and vascular disorders in the critical care setting.

**Supplementary material**

Supplementary material is available at \textit{British Journal of Anaesthesia} online.

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**Conflict of interest**

None declared.

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