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Hydrocortisone Preserves the Vascular Barrier by Protecting the Endothelial Glycocalyx

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Background: **Hydrocortisone protects against ischemia–reperfusion injury, reduces paracellular permeability for macromolecules, and is routinely applied in the prevention of interstitial edema. Healthy vascular endothelium is coated by the endothelial glycocalyx, diminution of which increases capillary permeability, suggesting that the glycocalyx is a target for hydrocortisone action.**

Methods: **Isolated guinea pig hearts were perfused with Krebs-Henseleit buffer. Hydrocortisone was applied in a stress dose (10 μg/ml) before inducing 20 min of ischemia (37°C). Hearts were reperfused for 20 min at constant flow (baseline perfusion pressure, 70 cm H_2O) with Krebs-Henseleit buffer or Krebs-Henseleit buffer plus 2 g% hydroxyethyl starch (130 kd). Coronary net fluid filtration was assessed directly by measuring transudate formation on the epicardial surface. Hearts were perfusion fixed to visualize the glycocalyx.**

Results: **Ischemia-induced degradation of the glycocalyx enhanced coronary perfusion pressure (118.8 ± 17.3 cm H_2O) and increased vascular permeability (8 ± 0.2 $\mu l \cdot min^{-1} \cdot cm\ H_2O^{-1}$ at baseline *vs.* 34 ± 3.3 $\mu l \cdot min^{-1} \cdot cm\ H_2O^{-1}$ after reperfusion). Enzymatic digestion of the glycocalyx (heparinase) elicited similar effects. Hydrocortisone reduced postischemic oxidative stress, perfusion pressure (86.3 ± 6.4 cm H_2O), and transudate formation (11 ± 0.6 $\mu l \cdot min^{-1} \cdot cm\ H_2O^{-1}$). Applying colloid augmented this (70.6 ± 5.6 cm H_2O and 9 ± 0.5 $\mu l \cdot min^{-1} \cdot cm\ H_2O^{-1}$). Postischemic shedding of syndecan-1, heparan sulfate, and hyaluronan was inhibited by hydrocortisone, as was release of histamine from resident mast cells. Electron microscopy revealed a mostly intact glycocalyx after hydrocortisone treatment, but not after heparinase treatment.**

Conclusions: **Hydrocortisone preserves the endothelial glycocalyx, sustaining the vascular barrier and reducing interstitial edema. The effect of colloids suggests that prevention of postischemic rise in coronary resistance by hydrocortisone could also be based on alleviation of endothelial swelling. Stabilization of myocardial mast cells by hydrocortisone may account for the mitigated inflammatory affect of ischemia–reperfusion.**

DESPITE widespread clinical use and importance of hydrocortisone for treatment of various illnesses, cellular and molecular mechanisms of action are not completely understood and are partially contradictory.[1]

It is known that hydrocortisone exhibits protective effects against ischemia–reperfusion injury by mediating nontranscriptional activation of endothelial nitric oxide synthase.[2] Above that, it prevents the migration of inflammatory cells from circulation to tissues by blocking the synthesis of various chemokines and cytokines.[3] Corticosteroids increase systemic vascular resistance and potentiate the vasoconstrictive responses of catecholamines and angiotensin II.[4] The underlying mechanisms may involve down-regulation of expression and activity of enzymes producing vasodilatory agents such as nitric oxide and prostacyclin.[4] Furthermore, glucocorticoids are known to act on intercellular junctions,[5] somehow achieving a decrease in transendothelial fluid flow[6] and paracellular permeability for macromolecules[7] and, thus, are routinely applied in the prevention of interstitial edema and swelling.[8] In view of these various interactions, we endeavored to take a closer look at the effect of corticosteroids in the specific setting of postischemic reperfusion damage to the heart.

Cardiac ischemia–reperfusion leads to functional disturbance of cardiomyocytes and coronary endothelium, the latter partly due to impairment of endothelium dependent coronary vasodilatation. Sequelae are myocardial stunning and coronary low reflow.[9] However, early manifestation of endothelial injury after ischemia consists in a disruption of the glycocalyx.[10,11]

A healthy vascular endothelium is coated by numerous and varied transmembrane and membrane-bound molecules.[12] The principal proteins of this glycocalyx are transmembrane syndecans and membrane-bound glypicans. Both contain bound heparan sulfate and chondroitin sulfate side chains.[11] Together with bound plasma proteins, hyaluronan and solubilized glycosaminoglycans, the glycocalyx forms the endothelial surface layer with a thickness of approximately 1 μm, evident as an exclusion zone for erythrocytes.[13] It is well recognized that enzymatic digestion with heparinase, pronase, or hyaluronidase but also exposure to tumor necrosis factor α or ischemia can reduce the thickness of the endothelial glycocalyx.[10] Its diminution increases capillary permeability, leading to tissue edema, suggesting that the glycocalyx acts as a competent barrier against passage of water and colloids.[14–16]

Looking at the targets and outlined modes of action of glucocorticoids makes it seem likely that there could be links between the actions of hydrocortisone and the endothelial glycocalyx.

We investigated this possibility in an isolated perfused heart model designed to facilitate detection of coronary vascular function related to the state of the glycocalyx. Parameters measured included coronary transudate (a direct measure of net fluid extravasation), coronary perfusion pressure (directly related to coronary flow resis-

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tance), colloid permeability, metabolic stress (myocardial formation of lactate, purines, and urate), shedding of major components of the glycocalyx (syndecan-1, heparan sulfate, and hyaluronan), and derived visualization of the glycocalyx by electron microscopy. Release of histamine from resident mast cells served to assess the inflammatory state of the postischemic hearts.

Our study shows, for the first time, that stabilization of the glycocalyx by hydrocortisone maintains the physiologic endothelial permeability barrier and prevents postischemic low reflow in the face of ischemic stress and reperfusion damage.

Materials and Methods

The investigation conforms to the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health. The study was approved by the officially installed independent ethics committee of the State of Bavaria (file No. 209.1/211-2531.3-3/99, Munich, Germany).

Heart Preparation

Hearts of male guinea pigs (aged 6–8 weeks, body weight 250–300 g) were isolated and perfused with a modified Krebs-Henseleit buffer (116 mM NaCl, 23 mM $NaHCO_3$, 3.6 mM KCl, 1.16 mM KH_2PO_4, 1.2 mM $CaCl_2$, 0.58 mM $MgSO_4$, 5.4 mM glucose, 0.3 mM pyruvate, and 2.8 U/l insulin, gassed with 94.6% oxygen and 5.4% carbon dioxide at 37°C, pH 7.40 ± 0.05) in a modified Langendorff mode as described previously.[12,17,18] The oxygen is physically dissolved, achieving a partial pressure of oxygen of 400–500 mmHg in the coronary arterial perfusate and, thus, guaranteeing adequate oxygenation.[19] The coronary perfusion rate was regulated to achieve a constant pressure of 70 cm H_2O during the equilibration phase and was then maintained throughout the reperfusion to monitor changes in coronary perfusion pressure. Coronary venous effluent was collected from the cannulated pulmonary artery after draining from the coronary sinus into the right atrium and ventricle. Transudate, a mixture of interstitial and lymphatic fluids formed by net filtration and appearing on the epicardial surface, was collected over timed intervals from the apex of the heart.

Experimental Protocols

Immediately after explantation and preparation of the hearts, an equilibration interval of 15 min was allowed to establish steady state conditions. Hydrocortisone (Hydrocortison; Pharmacia GmbH, Karlsruhe, Germany) was applied during the entire equilibration phase at a rate ensuring a steady state stress-dose level of 10 μg hydrocortisone/ml perfusate. Baseline measurements of coronary effluent and transudate were performed in the last 2 min before induction of 20 min of warm (37°C), global stopped-flow ischemia. Samples of effluent were also collected between minutes 0–5, 5–10, 10–15, and 15–20 after onset of reperfusion. Transudate samples were collected over 2-min intervals at 0–2, 2–4, 4–6, 6–8, 12–14, and 18–20 min after start of reperfusion. Therefore, during the first—decisive—8 min, all fluids coming from the heart were collected and quantified gravimetrically. Because the rate of transudate formation is pressure dependent,[12,17,20] we related it to the respective perfusion pressure established at the given flow rates in each heart.

The experimental groups are characterized in figure 1. Global warm ischemia was induced for 20 min without (group A, n = 9) or with (group B, n = 9) previous application of hydrocortisone. Alternatively, the glycocalyx was enzymatically degraded by means of heparinase, 10 U enzyme (heparinase type I; Sigma-Aldrich, Seelze, Germany) being applied in the course of 10 min after equilibration (group C, n = 8; group D, n = 8). In groups E and F (without and with hydrocortisone, respectively, n = 8 each), reperfusion was conducted in the presence of hydroxyethyl starch. This was achieved by infusing 6% hydroxyethyl starch (molecular weight, 130,000; degree of substitution, 0.4; Fresenius AG, Bad Homburg, Germany) for 20 min into the Krebs-Henseleit buffer perfusate at a rate of one third of the actual, total coronary flow. Over and above that, time control experiments were performed to evaluate the influence of equilibration and perfusion without ischemia–reperfusion injury (groups G and H, respectively, n = 6 each).

At the end of each experiment, both atria and the large vessels were cut away, and the ventricles were weighed at once (wet weight) and again after 24 h at 60°C (dry weight) to establish a wet-to-dry weight ratio. This served as a quantitative measure for formation of edema.

Determination of Glycocalyx Components, Hydroxyethyl Starch, Release of Lactate, Uric Acid, Purines, and Histamine

In all groups, samples of effluent were used for assessing shedding of syndecan-1 (CD-138), heparan sulfate, and hyaluronan as described in detail elsewhere.[18,20,21] Syndecan-1 concentrations were determined using an enzyme-linked immunosorbent assay (Diaclone Research, Besancon, France). This kit used a solid phase monoclonal B-B4 antibody and a biotinylated monoclonal B-D30 antibody raised against syndecan-1. The detection steps rely on streptavidin–horseradish peroxidase and tetramethylbenzidine as chromogens. To measure heparan sulfate, samples were concentrated with 10-kd-cutoff ultrafilters (Millipore, Billerica, MA) and aliquots used for an enzyme-linked immunosorbent assay (Seikagaku Corporation, Tokyo, Japan) based on two antibodies specific for heparan sulfate–related epitopes. Hyaluronan measurements were performed using an enzyme-linked immunosorbent assay (Echelon

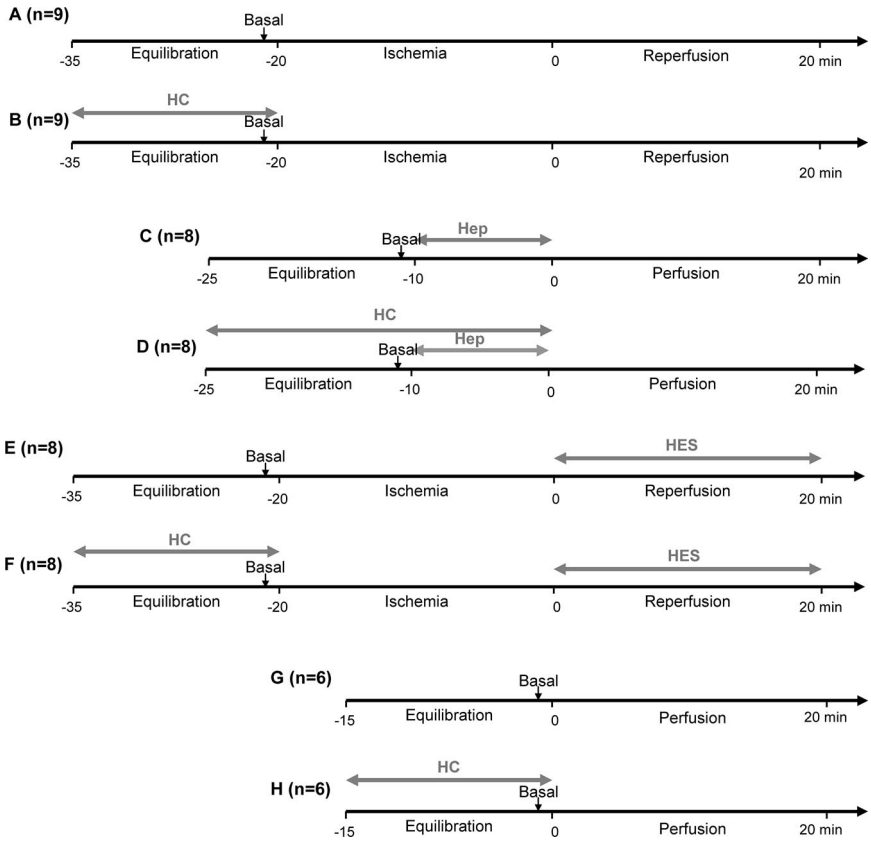

Fig. 1. Experimental protocols. Ischemia is defined as 20 min of no-flow warm ischemia, and (re)perfusion is defined as 20 min of constant-flow perfusion starting with a flow rate that had generated a perfusion pressure of 70 cm H_2O under basal conditions (equilibration). HC = hydrocortisone; Hep = heparinase; HES = hydroxyethyl starch.

Biosciences Incorporated, Salt Lake City, UT) based on an enzyme-linked antibody, after concentrating the samples with the aforementioned ultrafilters.

Concentrations of hydroxyethyl starch were quantified in the samples of coronary effluent and transudate using a method described by Förster *et al.*,[22] as modified by Rehm *et al.*[12] Basically, this involves hydrolysis to glucose and subsequent enzymatic/photometric determination.

Lactate, purines, and uric acid were determined by high-performance liquid chromatography in samples of coronary effluent. Lactate is an indicator of the severity of ischemic challenge. Purine release is directly related to the rate of energy consumption and inversely related to the rate of energy production. Uric acid, the end product of enzymatic purine metabolism in the heart, is subject to further oxidative chemical degradation. Therefore, hearts under oxidative stress typically release less urate relative to precursor purines than when oxidative stress is mitigated. For lactate, 10 μl supernatant was applied to a nucleosil 100-5NH_2 4 × 250-mm column (Macherey-Nagel, Dueren, Germany), 10 mM $NH_4H_2PO_4$, pH 3.5 served as eluent. Lactate was detected by its ultraviolet absorbance at 210 nm. Uric acid levels were determined according to Becker.[23] In brief, 10 μl supernatant was applied to a 5-μm C-18 nucleosil 4 × 250-mm column (Macherey-Nagel, Dueren, Germany). $HClO_4$–methanol–water at a ratio of 90:6:4 (vol%) served as eluent. Uric acid was detected by its ultraviolet absorbance at 280 nm. In the case of purines, all were transformed first to uric acid and then determined as such. This involved sequential enzymatic conversion of adenosine, inosine, hypoxanthine, and xanthine to urate.[23] To assess mast cell degranulation, histamine concentrations in samples of transudate were determined *via* enzyme-linked immunosorbent assay kit (Biotrend Chemicals GmbH, Cologne, Germany) according to the instruction manual.

Electron Microscopy

Electron microscopy was performed using a lanthanum fixation in modification of a method described by Vogel *et al.*[24] and Rehm *et al.*[12]

Statistical Analysis

Data dealing with rates of flow, transport, or release are expressed per gram heart weight. All data are presented as mean ± SEM, with n indicating the number of experiments. Comparisons involving two groups were made using the Mann–Whitney *U* test for independent data or the Wilcoxon test for dependent data. For comparisons of more than two groups, analysis of variance on ranks analysis was performed. *Post hoc* tests were performed using the Bonferroni method. $P < 0.05$ was considered to be significant. The statistical software used to conduct the analyses was SigmaStat 3.5 (Systat Software Inc., San Jose, CA).

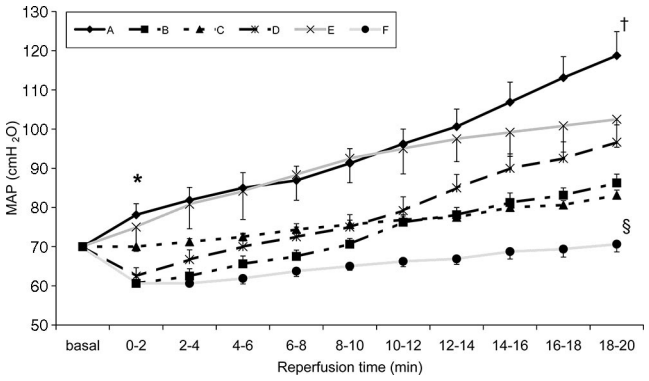

Fig. 2. Mean arterial coronary perfusion pressure (MAP) in relation to time during reperfusion. * Minute 2: A *versus* B, D, and F (H = 30.2, *df* = 5, *P* < 0.05). † Minute 20: A *versus* B, C, D, E, and F (H = 36.2, *df* = 5, *P* < 0.05). § Minute 20: F *versus* A, B, D, and E (H = 29.2, *df* = 4, *P* < 0.05).

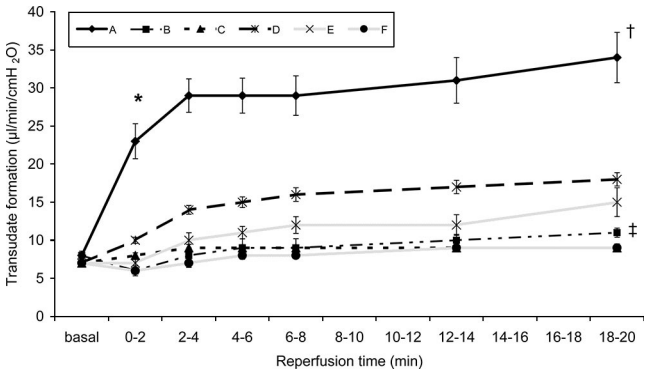

Fig. 3. Transudate formation in relation to sampling period during reperfusion. Transudate flow was normalized to perfusion pressure. * Minutes 0–2: A *versus* all groups (H = 41.3, *df* = 7, *P* < 0.05). † Minutes 18–20: A *versus* all groups (H = 42.6, *df* = 7, *P* < 0.05). ‡ Minutes 18–20: B *versus* A and E (H = 17.5, *df* = 2, *P* < 0.05).

Results

Perfusion Pressure

Coronary flow rate was adjusted during the equilibration phase to achieve a constant perfusion pressure of 70 cm H_2O. This individual baseline perfusion rate was then maintained during the entire, consecutive reperfusion phase. As shown in figure 2, coronary perfusion pressure in group A increased already within the first 2 min of reperfusion (78.1 ± 8.0 cm H_2O, W = −28, $P = 0.01$). This reflects an increase in coronary resistance. In contrast, there was an initial pressure decrease in all groups treated with hydrocortisone (group B: 60.6 ± 4.2 cm H_2O, W = −45, $P < 0.001$; group D: 62.5 ± 5.2 cm H_2O, W = −28, $P = 0.003$; group F: 60.6 ± 1.8 cm H_2O, W = −36, $P = 0.008$, minute 2 *vs.* basal), with no differences among the various hydrocortisone-treated groups. Decreases in perfusion pressure reflect coronary dilatation. With ongoing reperfusion, pressure increased significantly in all groups (groups A and B: W = 45, $P = 0.004$; groups C, D, E, and F: W = 36, $P = 0.008$, minute 2 *vs.* minute 20). However, coronary perfusion pressure and, thus, coronary resistance, were significantly higher in group A than in all other groups after 20 min (H = 36.2, *df* = 5, $P < 0.001$; pairwise *post hoc* tests: all $P < 0.05$; fig. 2).

Heparinase (group C) induced a significant pressure increase after 20 min compared with baseline (W = 36, $P = 0.008$). Pretreatment with hydrocortisone was unable to prevent this (group D).

Adding hydroxyethyl starch to the Krebs-Henseleit buffer perfusate after hydrocortisone treatment (group F) minimized the pressure increase after the initial decrease, perfusion pressure merely returning to the basal level after 20 min of reperfusion (70.6 ± 5.6 cm H_2O). In fact, perfusion pressure after 20 min of reperfusion was significantly lower compared with groups A, B, D, and E (H = 29.2, *df* = 4, $P < 0.001$; pairwise *post hoc* tests: all $P < 0.05$). Adding hydroxyethyl starch without hydrocortisone treatment produced no significant deviation *versus* the control group A during the entire reperfusion phase (group E).

However, time control measurements without ischemia also showed a slight increase in perfusion pressure with no difference between the control and hydrocortisone-treated groups (min 20; groups G and H: 78.8 ± 4.8 and 82.5 ± 2.9 cm H_2O, respectively, H = 5.4, *df* = 2, $P > 0.05$; results not shown).

Transudate Formation

Transudate formation, the direct measure of net fluid filtration in the intact coronary bed of the isolated heart, amounted to approximately 3% of coronary flow under basal conditions. Figure 3 presents transudate flow normalized to the individually pertaining perfusion pressure. The resulting baseline values measured during the equilibration phase showed no differences among the groups.

In the control group (group A), the formation of transudate increased approximately threefold within the first 2 min (W = 36, $P = 0.008$) and then further (fivefold) during the course of reperfusion. Pretreatment with hydrocortisone completely prevented the increase in coronary leak that developed during reperfusion (group B). Leak was also attenuated in the presence of colloid (hydroxyethyl starch, group E). Interestingly, the effects of hydrocortisone and colloid were additive, group F having the lowest transudate values of all postischemic hearts (fig. 3). Treatment with heparinase alone induced no significant increase in transudate formation. Hydrocortisone showed little effect after treatment with heparinase. If anything, transudate tended to be higher, with significant differences to both other hydrocortisone-treated groups (group D *vs.* groups B and F; H = 20.4, *df* = 2, $P < 0.05$). The final value of group A was significantly higher than in all other groups (H = 42.6, *df* = 7, $P < 0.05$). At the end of reperfusion, groups B and D showed significantly increased transudate formation *versus*

Table 1. Comparison of Metabolic Release during Reperfusion (n = 9 Each)

Release of	Time, min	Group A	Group B
Lactate,	Basal	1.63 ± 0.24	1.51 ± 0.18
μmol · min^{-1} · g^{-1}	0–5	10.92 ± 0.97*	10.98 ± 1.18*
	5–10	2.44 ± 0.22	2.49 ± 0.45
	10–15	2.05 ± 0.18	2.32 ± 0.34
	15–20	1.88 ± 0.16	2.06 ± 0.29
Purines,	Basal	3.25 ± 0.89	3.02 ± 0.63
nmol · min^{-1} · g^{-1}	0–5	334.75 ± 41.7*†	176.88 ± 15.4*
	5–10	67.25 ± 6.82*†	21.88 ± 8.77*
	10–15	33.01 ± 4.65*†	7.30 ± 4.18*
	15–20	23.63 ± 3.69*†	4.24 ± 2.24
Uric acid,	Basal	4.80 ± 0.78	4.26 ± 0.59
nmol · min^{-1} · g^{-1}	0–5	13.91 ± 1.72*	16.13 ± 0.91*
	5–10	6.89 ± 0.49	7.14 ± 0.67
	10–15	6.11 ± 0.49	4.50 ± 0.61
	15–20	5.77 ± 0.62	3.21 ± 0.43

Values are mean ± SEM.

* $P < 0.05$ *vs.* basal value. † $P < 0.05$, A *vs.* B.

Basal = value before ischemia under steady state conditions; group A = ischemia without pretreatment; group B = ischemia after pretreatment with hydrocortisone; minutes 0–5, 5–10, 10–15, and 15–20 = minutes after beginning of reperfusion; purines = sum of adenosine, hypoxanthine, inosine, and xanthine.

baseline (W = 28, $P = 0.01$ and W = 36, $P = 0.008$, respectively), whereas the hydrocortisone-colloid group (group F) showed no increase and no difference to both time control groups (group G: 7.2 ± 1.9 μl · min^{-1} · cm H_2O^{-1}; group H: 8.0 ± 2.1 μl · min^{-1} · cm H_2O^{-1}; results not shown).

Rates of Release of Lactate, Purines, and Uric Acid

Metabolic parameters of hearts subjected to ischemia–reperfusion are listed in table 1. Lactate release was identical in groups A and B during equilibration. There was a large increase in both groups after the start of reperfusion (both W = 45, $P = 0.004$), independent of the treatment. The rate of lactate release decreased rapidly during reperfusion (both W = 45, $P = 0.004$), returning to near basal values in both groups (table 1).

Basal purine release was identical in groups A and B. During the first minutes of reperfusion, purine release increased 50- to 100-fold (W = 36, $P = 0.008$), but the rate in group A was significantly higher than in group B (U = 2.0, $df = 8$, $P < 0.001$). Purine release decreased during reperfusion, returning to baseline values in the hydrocortisone-treated group B, whereas group A hearts maintained a higher release (U = 4.5, $df = 8$, $P = 0.002$), this also being significantly higher than the basal value (W = 36, $P = 0.008$; table 1).

Uric acid was released in comparable concentrations in group A and B hearts during equilibration. In the first minutes of reperfusion after ischemia, we measured a highly significant increase in uric acid release, with rates comparable between the groups (all W = 36, $P = 0.008$). After the peak, rates of release declined significantly to similar values after 10 min (table 1).

The ratio of urate release to release of precursor purines gives an indication of the degree of oxidative stress. For groups A and B, the basal release of urate was approximately equal to that of nonconverted precursor purines (ratio 1:0.7). In early reperfusion (minutes 0–5), purine release increased overproportionally, and attention should be drawn to a difference between group A (ratio 1:24) and group B (ratio 1:11), *i.e.*, the mismatch between urate and purines was only approximately half as great in the latter hearts. This indicates a relative reduction in oxidative stress in hearts reperfused after hydrocortisone treatment. Such an effect was still evident at 20 min of reperfusion (ratio 1:4.1 in group A and 1:1.3 in group B).

Extravasation of Hydroxyethyl Starch

Net colloid extravasation of hydroxyethyl starch was calculated as the product of transudate formation per gram heart weight and hydroxyethyl starch concentration in the transudate. Hearts subjected to ischemia without hydrocortisone (group E) showed a large increase of hydroxyethyl starch filtration from the beginning of reperfusion, filtration increasing further throughout the entire protocol (W = 28, $P = 0.01$). Colloid net filtration in the hydrocortisone-treated group F was significantly lower (W = −36, $P = 0.008$ *vs.* group E; table 2).

Measurement of Constituents of the Glycocalyx

Small amounts of CD-138 (syndecan-1)–positive material were detected in the effluent of all perfused hearts (basal value 144 ± 21 ng · min^{-1} · g^{-1}). As shown in figure 4A, ischemia–reperfusion enhanced shedding of

Table 2. Comparison of Parameters during Reperfusion (n = 9 Each)

Parameter	Time, min	Group A or E	Group B or F
Extravasation of HES,	Basal	ND	ND
mg · min^{-1} · g^{-1}	0–5	12.44 ± 1.23†	1.27 ± 0.28
	5–10	15.73 ± 2.14†	2.23 ± 0.49
	10–15	21.90 ± 2.41*†	2.41 ± 1.05
	15–20	29.42 ± 5.10*†	5.05 ± 0.58
Hyaluronan release,	Basal	2.75 ± 0.38	2.47 ± 0.29
μg · min^{-1} · g^{-1}	0–5	8.48 ± 2.01*†	3.92 ± 0.48
	5–10	3.95 ± 0.39	2.02 ± 0.23
	10–15	—	—
	15–20	—	—
Histamine release,	Basal	1.42 ± 0.15	1.49 ± 0.15
nmol · min^{-1} · g^{-1}	0–5	7.29 ± 1.29*†	0.86 ± 0.28
	5–10	8.52 ± 0.74*†	0.92 ± 0.23
	10–15	10.90 ± 1.02*†	1.09 ± 0.41
	15–20	11.93 ± 0.81*†	1.37 ± 0.42

Values are mean ± SEM.

* $P < 0.05$ *vs.* basal value. † $P < 0.05$, A *vs.* B.

— = not performed; basal = value before ischemia under steady state conditions; group A = ischemia without pretreatment; group B = ischemia after pretreatment with hydrocortisone; group E = ischemia without pretreatment adding hydroxyethyl starch (HES) during reperfusion; group F = ischemia after pretreatment with hydrocortisone adding HES during reperfusion; minutes 0–5, 5–10, 10–15, and 15–20 = minutes after beginning of reperfusion; ND = not detectable.

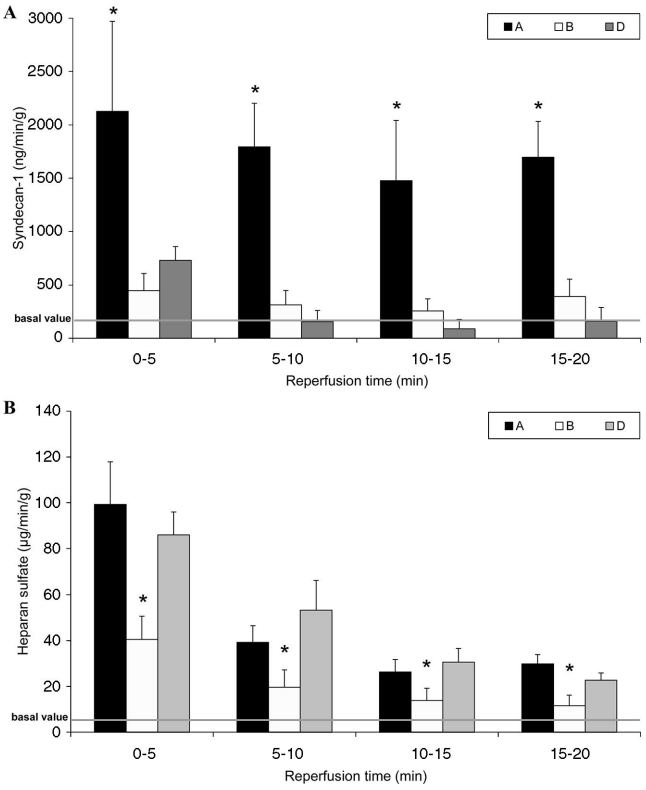

Fig. 4. Measurements of constitutional parts of the glycocalyx in coronary venous effluent. (*A*) Syndecan-1 release in relation to sampling period during reperfusion. (*B*) Heparan sulfate release in relation to sampling period during reperfusion. * A *versus* B and D ($P < 0.05$).

syndecan-1 15- to 20-fold. Application of hydrocortisone significantly decreased shedding at all times (group A *vs.* group B, minute 0–5: U = 116, $df = 9$, $P = 0.008$; minutes 5–10: U = 125, $df = 9$, $P < 0.001$; minutes 10–15: U = 113, $df = 9$, $P = 0.01$; minutes 15–20: U = 122, $df = 9$, $P = 0.001$). Notably, application of heparinase after hydrocortisone treatment (group D) elicited no increased shedding of syndecan-1 compared with group B ($P > 0.05$; fig. 4A).

Heparan sulfate was also detected in the effluent of all hearts (basal value 3.2 ± 1 $\mu g \cdot min^{-1} \cdot g^{-1}$). Ischemia–reperfusion induced strong shedding of heparan sulfate from the glycocalyx (fig. 4B). Application of hydrocortisone led to a significantly lower rate of release of heparan sulfate throughout reperfusion (group A *vs.* group B, minutes 0–5: U = 7.0, $df = 9$, $P = 0.007$; minutes 5–10: U = 9.0, $df = 9$, $P = 0.01$; minutes 10–15: U = 11.0, $df = 9$, $P = 0.03$; minutes 15–20: U = 7.0, $df = 9$, $P = 0.007$) but could not prevent shedding induced by application of heparinase (group B *vs.* group D, minutes 0–5: U = 58.0, $df = 8;9$, $P = 0.005$; minutes 5–10: U = 57.0, $df = 8;9$, $P = 0.007$; minutes 10–15: U = 55.5, $df = 8;9$, $P = 0.01$; minutes 15–20: U = 55.0, $df = 8;9$, $P = 0.02$; fig. 4B).

Hyaluronan release from the hearts increased transiently twofold to threefold after ischemia in group A (W = 34; $P = 0.01$), but no significant increase occurred in group B (table 2).

Electron Microscopy and Measured Tissue Edema

Electron microscopic photographs illustrating the state of the endothelial glycocalyx of coronary vessels are depicted in figure 5. No glycocalyx could be visualized in groups A, D, or E, but severe tissue edema was observed (figs. 5A and B show examples of a heart of group A). On the other hand, a mostly intact glycocalyx was seen in hydrocortisone-treated groups B and F with slight formation of edema (figs. 5C and D show examples of a heart of group B). The time control groups, G and H, that did not undergo ischemia or heparinase application also showed an intact glycocalyx (figs. 5E and F show examples of a heart of group G).

Mean wet-to-dry weight ratios of isolated hearts were 7.0 ± 0.7 and 7.4 ± 0.7 when measured after preparation and 45 min of perfusion with Krebs-Henseleit buffer with or without hydrocortisone treatment (time control groups G and H, respectively). The ratios were higher in all groups with a significant degradation of the glycocalyx, *i.e.*, after ischemia or heparinase application (group A: 8.6 ± 0.4; group C: 8.5 ± 0.6; group E: 8.5 ± 0.5, H = 21.1, $df = 4$, $P < 0.001$, pairwise *post hoc* tests: all $P < 0.05$ *vs.* groups G and H), indicating a significant increase in tissue edema formation. Application of hydrocortisone to the hearts before ischemia produced significantly lower ratios than in groups A, C, and E (group B: 7.2 ± 0.6; group D: 7.6 ± 0.4; group F: 7.0 ± 0.6, H = 28.4, $df = 5$, $P < 0.001$, pairwise *post hoc* tests: all $P < 0.05$ *vs.* groups A, C, and E), with no difference in edema compared with baseline.

Rates of Release of Histamine

Small amounts of histamine are released from resident mast cells of isolated perfused heart preparations already under basal conditions. However, release in ischemia–reperfusion control group A showed a significant increase after the start of reperfusion (W = 36, $P = 0.008$), increasing further throughout the reperfusion (W = 36, $P = 0.02$ *vs.* minutes 0–2). In contrast, hydrocortisone-treated group B showed no change in histamine release *versus* basal during the entire reperfusion phase (table 2).

Discussion

This study demonstrates, for the first time, the preventive impact of hydrocortisone on shedding of the endothelial glycocalyx in the situation of ischemia–reperfusion. The preservation of this structure should maintain the physiologic endothelial permeability barrier[14–16] and presumably prevent leukocyte[10,25,26] and platelet adhesion,[27] thereby mitigating inflammation and tissue edema. In com-

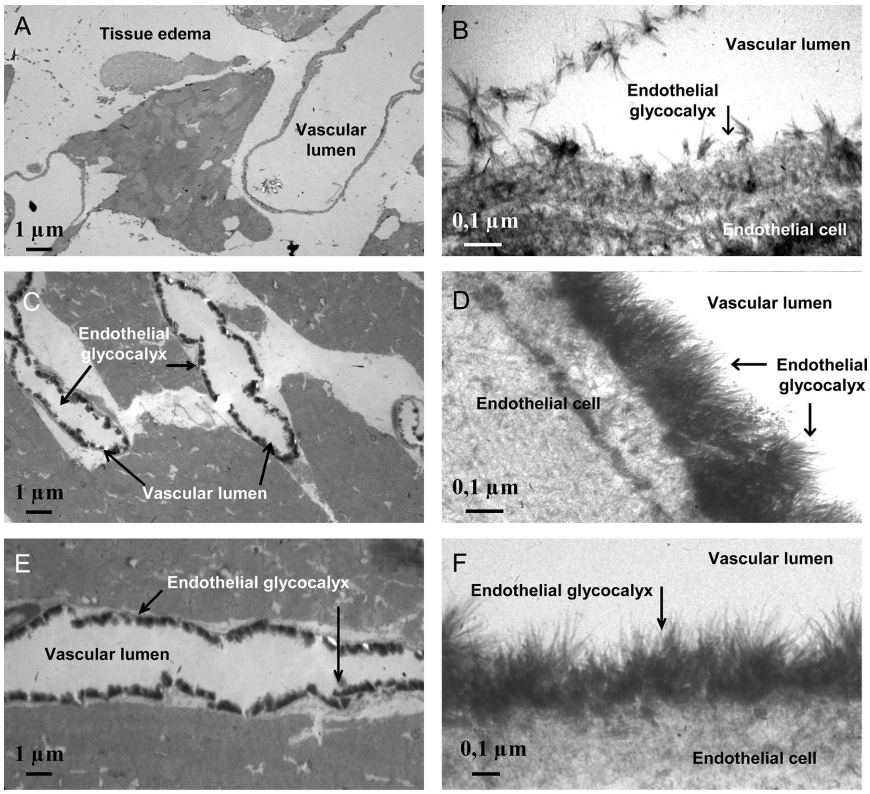

Fig. 5. Electron microscopic views of the hearts stained to reveal the glycocalyx. (*A* and *B*) Control experiment after 20 min of warm ischemia and reperfusion. The endothelial glycocalyx is nearly completely degraded, and a significant formation of edema is visualized. *A* is an overview, and *B* is a close-up view of the degraded glycocalyx. (*C* and *D*) After treatment with hydrocortisone and 20 min of warm ischemia and reperfusion. Endothelial glycocalyx is mostly intact, and less edema formation can be seen. *C* is an overview, and *D* is a close-up view of the intact glycocalyx. (*E* and *F*) Heart perfused for 35 min without ischemia. *E* is an overview, and *F* is a close-up view of the intact glycocalyx.

bination, such effects could alleviate the postischemic low reflow phenomenon.

Warm no-flow ischemia induces severe damage to the vascular endothelium in the heart, degrading the entire glycocalyx already at durations of 20 min. This results in (1) an impressive increase in formation of transudate, a direct measure of the coronary fluid leak, (2) increased colloid extravasation, (3) profound tissue edema, and (4) a significant increase in coronary perfusion pressure. Preliminary reports indicate that this damage also accounts for much of the postischemic increase in adhesion of inflammatory leukocytes.[28] Hydrocortisone treatment produced significant improvements of vascular leak, colloid extravasation, and hemodynamic features, along with signs of lower metabolic and oxidative stress, suggesting secondary effects on the myocardial and endothelial cells beyond a protection of the glycocalyx. Of mechanistic relevance may be the finding that hydrocortisone also abolished the postischemic release of histamine. This is derived from resident mast cells in the heart tissue and liberated upon excitation.[29] Because mast cells are also stores for numerous proteases (tryptase, chymase, mast cell protease 5, heparinase activity, *etc.*),[30] stabilization by hydrocortisone and prevention of degranulation should abrogate proteolytic damage to cardiac tissue, including the endothelial glycocalyx.

Measurements of syndecan-1 and heparan sulfate, constituent parts of the glycocalyx, in the coronary effluent show high concentrations after ischemic injury (15- to 30-fold increases). Hyaluronan is known to be among the first markers for glycocalyx shedding and to indicate a possible endothelial damage.[21,31] However, because it is not a major component of the glycocalyx, only transient twofold increases were observed upon reperfusion. In addition, the electron microscopic pictures reveal a complete destruction of the glycocalyx as a facet of damage resulting from ischemia–reperfusion. Hydrocortisone significantly decreased the shedding of the glycocalyx, as evidenced both by measurements of the components and by electron microscopy.

If the cardioprotection elicited by hydrocortisone depends on this preservation, degrading the glycocalyx by alternative means should preclude the benefit. Heparinase has previously been shown to enzymatically degrade the glycocalyx,[12,20] without having any further protease activity.[20] We found that hearts treated with heparinase still profited to some extent from hydrocortisone, because less edema was formed. Our measurements now reveal that heparinase specifically sheds heparan sulfate, but not the core protein syndecan-1. This means that the glycocalyx is still partly existent and allows hydrocortisone to exert some beneficial action on the coronary endothelial vasculature, also on hearts exposed to heparinase. At this stage we do not know which proteases are responsible for postischemic shedding of the glycocalyx. These could very well be mast cell derived or activated. Preliminary studies show increases in tryptase and cathepsin B activity in the myocardium, the former being a typical mast cell constituent.

Vascular permeability is regulated by a double barrier, with the endothelial glycocalyx acting as a competent component additional to the barrier formed by the endothelial cells themselves.[12,15,32–34] Disruption of only one of these two components, while not altering the other, leads only to a moderate increase of vascular permeability. For example, applying heparinase digests the glycocalyx without major impact on endothelial cell function.[12] On the other hand, histamine application increases the interendothelial cell gaps with no immediate impact on the glycocalyx. Singly, both interventions lead only to modest increases of vascular permeability. In contrast, treatment of hearts with histamine, after removing the glycocalyx *via* application of heparinase, strongly increases coronary leak.[12] Warm no-flow ischemia not only degrades the glycocalyx but also has a marked impact on the endothelial cells, therefore affecting both components of the double barrier. On the basis of our histamine concentration measurements, one may conclude that hydrocortisone stabilizes the resident mast cells, inhibiting the release of histamine and thus indirectly preventing the formation of endothelial cell gaps. However, we regard the effect of hydrocortisone on histamine release more as a marker of its action on mast cells than as a major causal action.

Action on the combined glycocalyx and endothelial cell barrier is better assessed in the presence of colloidal substances.[17] Colloids are able to establish an oncotic gradient at the endothelial surface, opposing fluid filtration.[20] Indeed, adding hydroxyethyl starch to the perfusate during reperfusion (group E) already attenuated coronary fluid leak. In the case of hearts treated with hydrocortisone before ischemia, the additional application of hydroxyethyl starch accentuated the protection further, with hearts achieving the same values during reperfusion as before ischemia.

Some aspects of the model used in this study must be critically addressed. Constant-flow instead of constant-pressure perfusion was chosen for the current experimental model to guarantee constant levels of infused substances, *e.g.*, hydrocortisone and hydroxyethyl starch. The use of "stress doses" of hydrocortisone as a preoperative treatment in high-risk cardiac surgical patients has been shown to reduce perioperative stress exposure, decrease chronic stress symptoms, attenuate systemic inflammation after cardiac surgery with cardiopulmonary bypass, and improve early outcome.[35,36] The literature "stress-dose concentration" corresponds to 10 μg/ml perfusate for the guinea pig heart model.

A methodologic peculiarity of the isolated heart model is the fact that perfusion with artificial media for more than 30 min causes tissue edema even in undamaged hearts. The addition of colloidal substances such as hydroxyethyl starch helps to alleviate this if the endothelial glycocalyx is intact. Preventing edema formation should forestall elevation of extramural pressure, *i.e.*, elevation of coronary resistance largely insensitive to vasodilators. Furthermore, postischemic swelling of endothelial cells should be alleviated by extracellular colloids. Hearts receiving both hydrocortisone and hydroxyethyl starch indeed exhibited no increase in postischemic coronary perfusion pressure.

Because the isolated hearts are perfused with a blood-free Krebs-Henseleit buffer solution, it is impossible to assess aspects of ischemia–reperfusion injuries that may be initiated and/or influenced by plasma-borne factors and formed constituents of blood. On the other hand, physiologic antioxidants of plasma are absent, perhaps accentuating damage by reactive oxygen species. Furthermore, the Langendorff heart preparation per definition excludes performance of pressure-volume work. Therefore, the recovery of myocardial pump performance was not directly assessable in the current model.

Further insight into the action of hydrocortisone is provided by the metabolic parameters. In the current model, the ischemic impact on the hearts must have been similar in all groups, irrespective of the pretreatment with hydrocortisone, because there were no differences in washout and production of lactate during the reperfusion phase. Purine release provides a sensitive method to evaluate myocardial energy metabolism relative to basal states, catabolism of adenine nucleotides to purines being more sensitive than measurement of high-energy phosphates. According to our results, the hearts pretreated with hydrocortisone benefit metabolically during early and later reperfusion (table 1). This effect could be indirectly related to the reduction of both edema formation and oxidative stress. To assess the latter, we determined myocardial release of urate.

Uric acid, a powerful physiologic antioxidant, is subject to chemical degradation. Therefore, it is released in lower amounts relative to purine precursors when oxidative stress is high. During the first minutes of reperfusion, there was seemingly no difference in release of urate between the groups. However, in relation to the sum of precursor purines adenosine, inosine, hypoxanthine, and xanthine, the release of urate increased more than twice in the hydrocortisone group B. Similarly, after 20 min of reperfusion, the hearts treated with hydrocortisone had basal values of urate release, but a urate: purine ratio approximately three times that of the untreated control group A. The data obtained here thus show far more uric acid avoids oxidative chemical destruction in the hydrocortisone-treated hearts throughout reperfusion. Accordingly, hydrocortisone helps to alleviate delayed oxidative damage in the reperfused organ, perhaps secondary to the stabilization of mast cells, a rich source of proinflammatory cytokines in acute repefusion.[29] Additional reasons for improvement could lie in retention of extracellular superoxide dismutase and the better mediation of shear stress–depen-

dent production of nitric oxide by an intact endothelial glycocalyx.

Known cardioprotective measures such as hypothermia and staged reperfusion to minimize oxidative stress should also protect the glycocalyx. For example, decreasing temperature slows down proteolytic enzymes. Managing reperfusion has the potential to decrease oxidative stress. This should presumably attenuate oxidation-mediated activation of matrix metalloproteases. It lessens activation of myocardial mast cells,[29] as do membrane stabilizing agents. All of these effects should result in less shedding of the endothelial glycocalyx.

We conclude that hydrocortisone has a multifactorial impact on the postischemic, reperfused heart. Preservation of the endothelial glycocalyx, perhaps *via* mast cell stabilization, and the consequential maintenance of the vascular barrier with prevention of interstitial edema imply a great potential in the therapy of ischemia–reperfusion injury and sepsis. Indeed, clinical trials have already indicated a preventive stress-dose application of hydrocortisone to improve patient outcome after coronary bypass operations.[35,36] Further studies on patients undergoing major operations associated with tissue ischemia and reperfusion are necessary to substantiate our findings. Preservation of the glycocalyx by hydrocortisone in the face of sepsis seems a promising extension.

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