Atrial natriuretic peptide induces shedding of endothelial glycocalyx in coronary vascular bed of guinea pig hearts

Dirk Bruegger,1,2 Matthias Jacob,1,2 Markus Rehm,1 Michael Loetsch,2 Ulrich Welsch,3 Peter Conzen,1 and Bernhard Friedrich Becker2

1Clinic for Anesthesiology, 2Institute of Physiology, and 3Department of Anatomy, Ludwig-Maximilian-University Munich, Munich, Germany

Submitted 7 March 2005; accepted in final form 15 June 2005

A healthy vascular endothelium is coated by a large variety of extracellular domains of membrane-bound molecules, which together constitute the glycocalyx. The principal proteins on the endothelial cell surface are transmembrane syndecans and membrane-bound glypicans that both contain bound heparan sulfate and chondroitin sulfate, i.e., exist in the form of proteoglycans (19). Syndecan, the most prevalent proteoglycan, has been shown to be shed under the influence of thrombin and epidermal growth factors, and its shedding can be mediated by G protein-coupled receptors and protein tyrosine kinase receptors and inhibited by tissue inhibitor of metalloproteinase type 3 (5, 18). Together with bound plasma proteins the glycocalyx forms the endothelial surface layer, an exclusion zone for erythrocytes with a thickness of ~0.4–0.5 μm (19, 20). It is well recognized that enzymatic digestion with heparinase (Hep), pronase, or hyaluronidase (19), but also exposure to oxidized lipoprotein (25) or tumor necrosis factor-α (9), can reduce the thickness of the endothelial glycocalyx. Diminution of the endothelial glycocalyx, however, increases capillary permeability to water, solutes, and colloids, leading to tissue edema (19, 23, 24).

The present study evaluated, for the first time, the impact of ANP on the endothelial glycocalyx. The experiments were conducted in an intact vascular bed, namely, the complete coronary system of isolated, perfused hearts (guinea pig Langendorff preparations) and provided functional data for the importance of the glycocalyx in preventing tissue edema. The impact of a low-dose infusion of ANP (10^{-9} M) with and without enzymatic degradation of the glycocalyx by means of heparinase application as well as of Hep application alone was investigated and combined with measurements of net fluid filtration and passage of hydroxyethyl starch (HES) and electron microscopic visualization of the glycocalyx. Moreover, we quantified components of the glycocalyx in the coronary effluent under the influence of ANP.

MATERIALS AND METHODS

The investigation conforms with the Guide for the Care and Use of Laboratory Animals published by the National Research Council (14). The protocols were approved by the officially installed, independent ethics committee of the State of Bavaria (equivalent of IACUC) for approval. Licensure of the investigator was obtained from the Government of Upper Bavaria (file no. 209.1/211-2531.3-399).

Heart preparation. Guinea pig hearts were isolated and perfused in a modified Langendorff mode (8). In brief, animals (male, wt 200–250 g) were stunned by neck dislocation, and immediately after

Address for reprint requests and other correspondence: M. Rehm, Clinic for Anesthesiology, Ludwig-Maximilians-Univ., Marchioninistr. 15, D-81377 Munich, Germany (E-mail: markus.rehm@med.uni-muenchen.de).

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
ATRIAL NATRIURETIC PEPTIDE AND ENDOTHELIAL GLYCOCALYX

opening of the thorax the hearts were arrested with ice-cold isotonic saline. The aorta was quickly cannulated, and the coronaries were perfused at constant aortic pressure (80 cmH2O) with a modified Krebs-Henseleit buffer (in mM: 116 NaCl, 23 NaHCO3, 3.6 KCl, 1.16 KH2PO4, 1.2 CaCl2, 0.58 MgSO4, 5.4 glucose, and 0.3 pyruvate, with 2.8 U/l insulin) gassed with 94.5% O2-5.5% CO2 at 37°C, pH 7.40 ± 0.05. Hearts were removed from the thorax and prepared as described previously (8, 23). Coronary venous effluent was collected from the pulmonary artery, which was cannulated and ended at a slightly negative hydrostatic pressure to preclude any coronary venous congestion. The rate of coronary flow was measured by means of a small-animal flowmeter (T106; Transonic Systems, Ithaca, NY) and registered online. Interstitial and lymphatic fluids formed by net filtration appeared at the epicardial surface and dripped off the apex of the heart. This so-called transudate was collected over timed intervals (29). Transudate was weighed by means of a precision scale. All samples were immediately frozen. To administer the 6% HES solution (mol wt 200,000 ± 25,000, degree of substitution 0.5; Fresenius, Bad Homburg, Germany), a small catheter, connected with an infusion pump (Perfusor Secura, Braun Melsungen, Melsungen, Germany), was inserted into the aortic feed line of the hearts. Additional infusion lines were inserted into the aortic line to infuse ANP (Sigma-Aldrich, St. Louis, MO) and Hep type 1 enzyme (Sigma-Aldrich).

Experimental protocols. Figure 1 illustrates the experimental protocols applied to four groups of six hearts each. In the first group, the impact of HES solution on transudate formation was studied. These hearts served as a control group. After an equilibration interval of 15 min, a 6% HES solution was infused continuously for 20 min at a rate constantly adjusted to one-third of the actual coronary flow, ensuring a steady-state coronary level of ~2 g HES/dl perfusate (Fig. 1A, Control group). In the second group, the infusion of HES was combined with the simultaneous application of 10−9 M ANP for 20 min (Fig. 1B, ANP group). Samples of transudate and effluent were taken at baseline (before HES infusion) and at 1, 3, 6, 8, 10, 15, and 20 min after the start of the HES infusion.

In two further sets of experiments the glycocalyx was enzymatically degraded by means of Hep, 10 U of enzyme (heparinase type I) being applied over the course of 15 min. In the third group of hearts, after enzymatic degradation of the glycocalyx the infusion of HES was combined with the simultaneous application of 10−9 M ANP (Fig. 1C, ANP + Hep group). In the fourth group, immediately after the enzyme application HES was infused for 20 min, again at a rate of one-third of the actual coronary flow (Fig. 1D, Hep group). Transudate and effluent samples were obtained at the above-mentioned time points in the ANP + Hep and Hep groups. At the end of each experiment ventricular wet weight was determined by means of a precision scale.

Determination of HES. The concentration of HES was quantified in the samples of coronary effluent and transudate with a method described by Förster et al. (6), as modified by Rehm et al. (21, 23). The kinetics for appearance of HES in transudate was analyzed by calculating the time required for HES to reach half-maximum values (t½) of concentration in the transudate for each individual heart.

Determination of components of glycocalyx. In all groups, samples of effluent collected at minutes 6, 10, 15, and 20 after the start of HES infusion were used for assessing shedding of syndecan-1 (CD-138). Syndecan-1 concentrations were determined with an enzyme-linked immunosorbent assay (Diaclone Research, Besancon, France). This kit uses a solid-phase monoclonal B-B4 antibody and a biotinylated monoclonal B-D30 antibody raised against syndecan-1. The epitope of B-B4 has been localized between amino acids 90 and 93 within the extracellular domain of syndecan-1 (4). The detection steps included streptavidin-horseradish peroxidase and tetramethylbenzidine as chromogens.

Electron microscopy. Electron microscopy was performed with modification of a method described by Vogel et al. (27). At the end of the perfusion protocol, the aorta was perfused with a fixation solution containing 2% glutaraldehyde, 2% sucrose, 0.1 M sodium cacodylate phosphate, and 2% lanthanum nitrate. When the contractions of the hearts stopped (usually within 1 min), the hearts were submersed in the fixation solution described above. The left ventricular wall was diced immediately into 0.5- to 1-mm3 pieces, and the pieces remained immersed in the fixation solution for 2 h at 20°C. Thereafter, the pieces of myocardium were washed in a solution mixed from 18 ml of NaOH (0.1 N), 27 ml of H2O2, and 9 ml of sucrose (12%). Three of five blocks randomly selected from each heart were used for electron microscopy. After being rinsed in phosphate buffer containing 2% osmium tetroxide and 2% lanthanum nitrate for 2 h, the pieces were dehydrated in ethanol and embedded in Araldite. For orientation, semithin sections were stained with toluidine blue. Ultrathin sections (thickness ~45 nm) were cut on a Reichart ultramicrotome and stained with lead citrate (saturated solution) and uranyl acetate (in

Fig. 1. Experimental protocols. After heart preparation and an equilibration period of 15 min, 6% hydroxyethyl starch (HES) solution was infused for 20 min at a rate constantly adjusted to one-third of the actual coronary flow. This occurred in the absence (Control group, A) and presence (ANP group, B) of atrial natriuretic peptide (ANP). In 2 further series, hearts were pretreated with heparinase applied over the course of 15 min, followed again by the infusion of 6% HES in the presence (ANP + Hep group, C) and absence (Hep group, D) of ANP. HES, 2% HES solution; ANP, 10−9 M ANP; Hep, 10 U heparinase. Transudate and effluent samples were taken before (baseline) and 1, 3, 6, 8, 10, 15, and 20 min after the start of the HES infusion.

A Control
Heart preparation
Equilibration
Infusion (20 min)
-15 baseline 0 20
min
B ANP
Heart preparation
Equilibration
Infusion (20 min)
HES + ANP
-15 baseline 0 20
min
C ANP + Hep
Heart preparation
Equilibration
Infusion (20 min)
HES + ANP
-30 baseline -15 0 20
min
D Hep
Heart preparation
Equilibration
Infusion (20 min)
HES
-30 baseline -15 0 20
min
70% methanol). During electron microscopic examination (Philips CM10), whole capillary profiles were photographed systematically so that all capillaries in a section were photographed, with the exception of those that were collapsed. This was justified because only the capillaries with an open lumen were uniformly stained with the glycocalyx tracer and only in these was it possible to assess the thickness of the layer. The photographed capillaries presented really reflect the general and fairly uniform changes seen in the respective perfusion groups. Black-and-white images of ~50 capillaries of at least 2 hearts of each group were compiled. Electron microscopic views were assessed qualitatively only.

Statistical analysis. Data dealing with rates of flow, transport, or release are expressed per gram of heart weight. All data are presented as means ± SE, with n indicating the number of experiments. For normally distributed data, comparisons were made by analysis of variance with the Bonferroni correction. For data that were not normally distributed, comparisons were made by analysis of variance on ranks for multiple comparisons. Post hoc testing was performed with the Student-Newman-Keuls method for multiple comparisons. A P value <0.05 was considered to be significant.

RESULTS

Baseline transudate formation, i.e., the direct measure of net fluid filtration in the intact coronary bed, and baseline coronary effluent flow of isolated guinea pig hearts are listed in Table 1. Transudate flow amounted to ~2–3% of coronary flow (Table 1) and did not differ significantly among the four groups. Figure 2 illustrates changes in transudate formation with time for all experimental groups. In the Control and Hep groups, transudate formation did not change significantly with respect to baseline (~13% and ~14% after 20 min, respectively). In contrast, in the ANP and ANP+Hep groups, transudate formation increased significantly versus basal, the rise amounting to approximately +29% and +31% after 20 min, respectively. Moreover, we observed a significant increase in transudate formation at minutes 15 and 20 in the ANP and ANP+Hep groups versus the Control group (Fig. 2). Changes in coronary effluent flow, on the other hand, were slight and, above all, comparable in all experimental groups; there were no intra- or intergroup differences (Fig. 3).

In all groups, the concentration of HES in the coronary effluent increased to steady-state values of almost 2 g/dl within 4 min (data not shown). Time courses for HES in the transudate related to the respective concentration in the coronary effluent are presented in Fig. 4. Compared with the Control group, HES was significantly higher in transudate in the Hep group at minute 10, in the ANP group at minutes 3, 6, and 10, and in the ANP+Hep group at minutes 1, 3, 6, and 10. This accelerated extravasation of colloid was reflected by the values for t½: 3.5 ± 0.6 min in ANP, 3.3 ± 0.6 min in ANP+Hep, and 3.8 ± 0.3 min in Hep, all significantly lower than the Control value of 5.6 ± 1.4 min (P < 0.05).

Net colloid extravasation was calculated as the product of transudate formation and HES concentration in transudate and is shown in Fig. 5. Compared with Control hearts, net HES extravasation was significantly higher in ANP+Hep and Hep.

Table 1. Baseline values for transudate formation and coronary effluent flow

<table>
<thead>
<tr>
<th>Group</th>
<th>Transudate Formation, ml/min/g</th>
<th>Coronary Effluent Flow, ml/min/g</th>
<th>Transudate, % coronary effluent flow</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.099 ± 0.017</td>
<td>4.4 ± 0.5</td>
<td>2.4 ± 0.6</td>
</tr>
<tr>
<td>ANP</td>
<td>0.110 ± 0.006</td>
<td>5.2 ± 0.5</td>
<td>2.5 ± 0.2</td>
</tr>
<tr>
<td>ANP+Hep</td>
<td>0.129 ± 0.015</td>
<td>4.8 ± 0.4</td>
<td>2.8 ± 0.4</td>
</tr>
<tr>
<td>Hep</td>
<td>0.136 ± 0.023</td>
<td>4.7 ± 0.5</td>
<td>2.8 ± 0.5</td>
</tr>
</tbody>
</table>

Data are means ± SE; n = 6 hearts for all groups. Groups are as defined in Fig. 1. ANP, atrial natriuretic peptide; Hep, heparinase.

Fig. 2. Changes in transudate formation vs. baseline value. Baseline values are given in Table 1; groups are as defined in Fig. 1. Transudate flow was determined at baseline and after the start of the HES infusion. Values are means ± SE. *P < 0.05, intergroup difference vs. Control group; **P < 0.05, intragroup difference vs. baseline.

Fig. 3. Changes in coronary effluent flow vs. baseline. Baseline values are given in Table 1; groups are as defined in Fig. 1. Coronary effluent flow was measured at baseline and after the start of the HES infusion. Values are means ± SE.
hearts at minute 10 and in ANP, ANP+Hep, and Hep hearts at minutes 15 and 20 ($P < 0.05$).

Interestingly, CD-138-positive material was detected in the coronary effluent of the isolated, perfused hearts. The quantitative release of CD-138 (syndecan-1) in the effluent is depicted in Fig. 6 for all groups. Application of ANP alone or after Hep increased shedding of syndecan-1 significantly at all measured time points compared with Control hearts. This was not the case for hearts pretreated with Hep alone, despite a tendency for increased release here, too. Thus ANP rapidly induced a shedding of syndecan-1.

Electron microscopic photographs illustrating coronary vessels of the isolated guinea pig hearts are shown in Fig. 7. An endothelial glycocalyx of ∼200- to 300-nm thickness was observed in the Control group (Fig. 7, A and B). In the ANP group, the endothelial glycocalyx appeared somewhat frayed in microvascular segments (Fig. 7, C and D). After Hep pretreatment [ANP+Hep (Fig. 7, E and F) and Hep (Fig. 7, G and H)] nearly no glycocalyx could be visualized by electron microscopy.

**DISCUSSION**

The main finding of the present study was an alteration of the integrity of the endothelial glycocalyx due to the application of ANP associated with both an increased transudate formation and an enhanced extravasation of colloid. There have been previous reports that the release of ANP enhances extravasation of fluids and macromolecules (2, 7, 12, 15, 16, 30). However, in all these investigations, the mechanism for the permeability-increasing effect of ANP remained undefined.

In our study we compared the action of ANP functionally and histologically with that of Hep. First, compared with Control hearts, net fluid filtration (transudate) increased significantly after enzymatic degradation of the glycocalyx by means of Hep application. The application of ANP ($10^{-9}$ M) also led to a significant increase in fluid extravasation independent of pretreatment with Hep (Fig. 2). Pertinently, these changes in transudate formation were not related to any changes in coro-
nary effluent flow (Fig. 3). Second, in hearts exposed to ANP, ANP with Hep, or Hep alone not only was an increase in fluid leak observed but also an enhanced passage of HES into the interstitial fluid (cf. Figs. 4 and 5). Third, the quantification of syndecan-1, a component of the endothelial glycocalyx, by means of syndecan-1 measurement in the coronary effluent and electron microscopic visualization of the glycocalyx suggests that ANP induces degradation of the glycocalyx: compared with Control hearts, syndecan-1 release in the coronary effluent was significantly higher in ANP and ANP + Hep hearts (Fig. 6). Visualization of the endothelial glycocalyx showed a mostly intact glycocalyx in the Control group. Surprisingly, ANP alone (without heparinase application) led to a distinct alteration of the glycocalyx. As readily expected, no La<sup>3+</sup>-stainable glycocalyx remained after enzymatic pretreatment with heparinase (Hep and ANP + Hep, Fig. 7).

Tissue fluid balance, plasma volume regulation, and edema formation are governed by the Starling principle of microvascular fluid exchange. However, this hypothesis was born without any knowledge of an endothelial glycocalyx. Recent theoretical and experimental investigations showed that local colloid osmotic and hydrostatic pressures directly behind the endothelial glycocalyx (tissue side of the glycocalyx in the cleft between adjacent endothelial cells) are much lower than the respective tissue colloid osmotic and tissue hydrostatic pressures (1, 10, 11). Moreover, Michel (17) and Weinbaum (28) proposed that the effective osmotic barrier is not the whole capillary wall but the luminal glycocalyx, which acts as a primary molecular filter. This concept introduces an important difference between the global Starling forces across the entire endothelial layer and those across the glycocalyx. For instance, after 20 min of HES infusion, the concentration of HES in the transudate approximated that in the effluent (Fig. 4). At first glance, one would argue that no effective oncotic difference exists anymore across the whole capillary wall. Nevertheless, we observed an enduring reduction in transudate formation.

Fig. 7. Electron microscopic views of hearts stained to reveal the glycocalyx (representative of 2 hearts each). A and B: infusion of HES (Control group). C and D: simultaneous infusion of HES and ANP (ANP group). E and F: simultaneous infusion of HES and ANP after enzymatic pretreatment with heparinase (ANP + Hep group). G and H: infusion of HES after enzymatic pretreatment with heparinase (Hep group).
during HES infusion in Control hearts: after the start of infusion of HES at a coronary level of ~2 g HES/dl perfusate, transudate formation decreased persistently by ~15% (Fig. 2). According to Adamson et al. (1), in the presence of an intact endothelial glyocalyx the effective oncotic forces are those in the intravascular and subglyocalyx spaces. Thus an increase in the oncotic pressure within the vascular lumen brought about by the infusion of HES as opposed to a small oncotic force directly behind the glyocalyx would explain our findings nicely.

The remaining three groups (ANP, ANP+Hep, and Hep) were all characterized by an altered, largely destroyed endothelial glyocalyx. The damage to the molecular barrier was reflected by a more rapid extravasation of HES compared with the group with an intact glyocalyx (Control). Consequently, in these groups, the effective oncotic forces are predominantly the intravascular and interstitial colloid osmotic pressures. The biphasic time courses of change in transudate formation observed here (Fig. 2) are consistent with the model proposed. Initially, because of the infusion of HES, there was a high intravascular colloid osmotic pressure and transudate formation tended to decrease in all groups. This initial decrease in transudate formation was only maintained in the group with an intact glyocalyx (Control). In groups with an altered endothelial glyocalyx, transudate formation began to increase after ~3–6 min, in line with shedding of the glyocalyx and the appearance of HES in the interstitial compartment, i.e., with a decreased effective oncotic pressure difference opposing filtration. Moreover, in these groups the damaged endothelial glyocalyx is less of a permeability barrier to water, which may also contribute to the augmented extravasation of fluid. This interpretation is justified because the surface area of the perfused microvessels should not have changed, coronary flow and perfusion pressure remaining constant in all groups. Alternatively, the difference in time course in the rise of transudate HES concentration between the Control and experimental groups could be accounted for by lower transudate formation and consequently slower washout of the tissue spaces in the Control group compared with the experimental groups.

Our finding that the concentration of HES in transudate rose to levels near those in coronary effluent after 20 min of continuous HES infusion (Fig. 4) would seem to indicate that the exchange vessels of our preparations are rather leaky. This is not unexpected, because it is well known that perfusion with solutions lacking certain plasma proteins (e.g., albumin and orosomucoid) increases the permeability of most vascular beds. However, we are able to rule out contamination of transudate with coronary effluent. In previous experiments (unpublished observations) in which we added washed guinea pig red blood cells to the arterial perfusate, we never observed red blood cells in transudate of intact preparations with tightly ligated cardiac veins and arterial cannuulas. To heighten sensitivity, the samples of transudate had been spun in Eppendorf tubes to concentrate any red blood cells as a sediment. After the supernatant was decanted no hemoglobin was detected via the cyanohemoglobin assay. Thus we consider transudate to be a mixture of interstitial and lymphatic fluids. It is certainly no measure of the pure ultrafiltrate expected to be formed in the arteriolar and capillary segments of an intact coronary bed with their “continuous” type of endothelial lining. In our opinion, the chief determinant of transudate formation and colloid exchange may well be localized to the venular segments of the coronary system. Indeed, histochemical examinations of the hearts in our study after brief luminal application of marker (La³⁺) suggest the leak to be in the vicinity of very thin-walled vessels (just endothelium without any smooth muscle or pericytes) of ~15- to 30-μm diameter, i.e., presumably “large-pore” venular segments of the coronary bed.

The present observations are in good agreement with other studies elucidating the role of the endothelial glyocalyx for vascular permeability (3) and highlighting its extraordinary thickness (26). A change in vascular permeability after enzymatic digestion of the glyocalyx was investigated by Huxley and Williams (13), using coronary arterioles isolated from pigs. After Hep application, an increase in permeability for α-lactalbumin and albumin was found (13). In addition, van den Berg et al. (24) demonstrated that degradation of the endothelial glyocalyx from the coronary endothelial surface of isolated rat hearts resulted in notable myocardial tissue edema. These facts together with the functional data of the present study indicate that the ANP-induced increase in transudate formation might be at least partially related to changes in the integrity of the endothelial glyocalyx.

Other changes brought about by ANP might also contribute to the observed enhanced extravasation of fluids and HES. For instance, the slit pore and the vesicles could respond to ANP, possibly via cGMP-mediated signal cascades. However, from previous studies (unpublished observations) in our laboratory we know that the leak-enhancing action of ANP is not fully reversible, even after 30 min of washout, which speaks for a “structural” modification. Nonetheless, the changes seen in our model must be interpreted cautiously. At this stage, they cannot be generalized to the situation in vivo or to organs other than the heart.

It is evident from the present data that ANP induces shedding of syndecan-1, but how this is regulated remains unknown. Receptor-mediated activation of metalloproteinases seems a possibility. Syndecan-1 has been shown to be shed after application of thrombin and epidermal growth factors, and its shedding can be mediated by G protein-coupled receptors and protein tyrosine kinase receptors and inhibited by tissue inhibitor of metalloproteinase type 3 (5). One additional finding of the present study deserves mention. There seems to be an inconsistency between the failure to detect significant amounts of syndecan-1 in the coronary effluent of hearts treated with heparinase alone (Fig. 6) and the absence of an endothelial glyocalyx in the electron microscopic views of these preparations (Fig. 7, G and H). However, syndecan-1, as detected by the antibody used here, is the core protein to which the heparan sulfate groups are attached. Cleavage of heparan residues by Hep treatment should leave the core protein intact and anchored to the cell membrane. This residual covering does not bind La³⁺ efficiently, and so, as judged by electron microscopy, there is hardly any effective glyocalyx left.

The results of the present study might help to explain the findings of previous double-label blood volume measurements in patients (21, 22). With the same infusion solution used here (6% HES; mol wt 200,000, degree of substitution 0.5), ~60% of the infused volume had left the intravascular space 30 min after volume loading (21). Moreover, considerable amounts of the HES extravasated quickly (21). Most likely, volume loading will result in increased levels of plasma ANP in humans.
Should this initiate degradation of the glycocalyx, increased net fluid filtration and extravasation of colloid are to be expected. Furthermore, in the double-label blood volume measurements, a greater decrease in arterial blood hematocrit (measured by centrifugation of arterial blood samples) in relation to whole body hematocrit (derived by double-label measurements of erythrocyte and plasma volume) was found during volume loading (21). The latter observation leads one to suspect that a considerable decrease in the volume of the endothelial surface layer occurred during the volume loading, leading to a larger distribution space for the red blood cells. Again, the data of the present study suggest that release of ANP may be the underlying cause of the alteration of the endothelial glycocalyx.

In summary, the present study shows that individual components of the glycocalyx may be shed after application of ANP and that ANP induces histologically detectable degradation of the glycocalyx. Thus the ANP-induced increase in permeability described in vivo might be at least partially related to changes in the integrity of the endothelial glycocalyx.

REFERENCES


