Heparin Binding Protein in Adult Heart Surgery

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Background. **Heparin binding protein (HBP) is released from neutrophilic secretory vesicles upon neutrophil adhesion on the endothelium. HBP mediates capillary hyperpermeability experimentally. In sepsis, HBP predicts organ dysfunction. Cardiopulmonary bypass induces neutrophil activation and hyperpermeability. We hypothesized that in cardiopulmonary bypass, HBP is released in the reperfused coronary circulation concomitantly with neutrophil adhesion.**

Methods. **In 30 patients undergoing aortic valve replacement, concomitant blood samples were drawn from the coronary sinus and arterial line before aortic cross-clamping and 5 minutes after reperfusion to calculate transcoronary differences. Plasma HBP concentrations, neutrophil markers lactoferrin and myeloperoxidase, myocardial injury marker heart-type fatty acid binding protein, and leukocyte differential counts were measured.**

Results. **Arterial HBP was 4.1 ng/mL (interquartile range [IQR], 3.6 to 5.3 ng/mL) preoperatively and 150.0 ng/mL (IQR, 108.2 to 188.6 ng/mL) after aortic declamping. HBP increased 39-fold, lactoferrin 16-fold, and myeloperoxidase fourfold during cardiopulmonary bypass. Before cardiopulmonary bypass, there were marginal transcoronary differences in HBP (1.4 ng/mL; IQR, –0.4 to 3.6 ng/mL; $p = 0.001$) and heart-type fatty acid binding protein (0.4 ng/mL; IQR, –0.04 to 3.5 ng/mL; $p = 0.001$) but not in the other indicators. During reperfusion, transcoronary HBP release (6.4 ng/mL; IQR, 1.8 to 13.7; ng/mL; $p < 0.001$) was observed concomitantly with transcoronary neutrophil sequestration (–0.14 × 10^9/L; IQR, –0.28 to 0.01 × 10^9/L; $p = 0.001$) and transcoronary heart-type fatty acid binding protein release (6.9 ng/mL; IQR, 3.0 to 25.8 ng/mL; $p < 0.001$). There were no transcoronary differences in lactoferrin or myeloperoxidase during reperfusion.**

Conclusions. **Cardiopulmonary bypass results in substantial increase in circulating HBP. HBP is also released from the reperfused coronary circulation concomitantly with coronary neutrophil adhesion and myocardial injury. HBP may be one candidate for a humoral factor mediating capillary leak in cardiopulmonary bypass.**

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Heparin binding protein (HBP) is a 37-kD granule protein of neutrophils. In neutrophils, it is located in the secretory vesicles and the azurophilic granules [1]. This dual localization makes HBP an interesting biomarker for neutrophil activation. Because azurophilic granules show the lowest propensity to be exocytosed, HBP stored in these granules is mainly released in the tissues after extravasation. Secretory vesicles, however, are mobilized first [2]. This results in a rapid increase in plasma HPB concentrations. As a sensitive indicator of intravascular neutrophil activation, HBP has gained increasing interest as a promising inflammatory biomarker in recent years. High HBP is associated with severe sepsis and septic shock [3]. Importantly, HBP performs well in predicting meaningful clinical outcomes. Among septic patients, it predicts development of organ dysfunction in general [4] as well as more specifically circulatory failure [5, 6], respiratory failure [6, 7], and acute kidney injury [8, 9]. After cardiac arrest, HBP concentrations predict death [10].

Neutrophils are activated upon adhesion to endothelial cells. During firm adhesion, coupling of β_2-integrins on neutrophil plasma membrane triggers release of HBP [11]. Intraluminally released HBP binds to glycosaminoglycans on the endothelium [12, 13]. HBP is proposed to have an important role in capillary leak. It induces endothelial hyperpermeability in vitro [11, 14]. In experimental conditions in vivo, intravenous administration of HBP induces acute lung injury with histologic features similar to those after lipopolysaccharide administration [14]. Finally, in patients with septic shock, plasma HBP concentrations are associated with fluid overload and the degree of hypoxemia [14]. Like sepsis, cardiopulmonary bypass (CPB) is also associated with hyperpermeability [15, 16].

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Abbreviations and Acronyms

CPB = cardiopulmonary bypass
HBP = heparin binding protein
hFABP = heart-type fatty acid binding protein
IQR = interquartile range
LF = lactoferrin
MPO = myeloperoxidase
Po_2 = partial pressure of oxygen
So_2 = oxygen saturation

A substantial amount of data demonstrate pathophysiological significance of activated neutrophils in experimental cardiac ischemia-reperfusion injury [17]. Although CPB is classically known to result in strong neutrophil activation, coronary neutrophil sequestration after aortic declamping has not been associated with concomitant transcoronary neutrophil activation in cardiac surgical patients [18]. Intracoronary neutrophil activation upon endothelial adhesion has probably been beyond the detection limit of available laboratory methods. HBP has not been investigated in cardiac surgery.

The present study had two hypotheses. First, we hypothesized that CPB would induce a more pronounced increase in HBP than in classical markers of neutrophil-specific granules (lactoferrin [LF]) and azurophilic granules (myeloperoxidase [MPO]) [19]. Second, we hypothesized that HBP would be sensitive enough to detect intracoronary neutrophil activation during reperfusion after aortic declamping in patients without coronary artery disease undergoing aortic valve reconstruction.

Patients and Methods

The study was approved by the Helsinki University Hospital Ethics Committee. Written informed consent was obtained from all patients before enrollment in the study. We prospectively recruited 30 patients undergoing aortic valve replacement operations because of aortic valve stenosis. Exclusion criteria were coronary artery disease, left ventricular ejection fraction of less than 0.30, systemic glucocorticoid medication, or need for perioperative glucocorticoid substitution, immunosuppressive medication, cardiac operation other than aortic valve replacement in the same session, atrial fibrillation, and insufficient cessation of antiplatelet and anticoagulation therapy (clopidogrel or ticagrelor <5 days, low-molecular-weight heparins <2 days). Anesthesia was induced with etomidate, alfentanil, and rocuronium and maintained with sevoflurane, alfentanil infusion, and rocuronium boluses. A pulmonary artery catheter was placed after the induction of anesthesia.

Affinity NT oxygenator (Medtronic International Trading Sàrl, Tolochenaz, Switzerland) oxygenator and Trillium (Medtronic International Trading Sàrl) tubing were used. Patients received 300 IU/kg heparin and additional boluses to achieve an activated clotting time exceeding 480 seconds. The aorta and right atrium were cannulated. The coronary sinus was cannulated with a 14F balloon-tipped Retrograde Cardioplegia Catheter (Edwards Lifesciences, Irvine, CA). The initial volume of the antegrade cold blood cardioplegia solution (4:1 cardioplegia solution to blood ratio) was double the volume needed for cessation of all cardiac electrical activity but never less than 1,000 mL. Cardiac arrest was maintained by retrograde infusion of 300 mL of blood cardioplegia solution (8:1 cardioplegia solution to blood ratio) every 20 minutes.

A Stöckert S5 roller pump (Sorin Group Deutschland GmbH, Munich, Germany) was used for CPB, flow adjusted to 2.4 L/min × body surface area, mixed venous oxygen saturation maintained over 70%, fraction of inspired oxygen at 70%, partial pressure of carbon dioxide within 4.5 to 5.5 kPa, and mean arterial pressure at 40 to 60 mm Hg. Patients were moderately cooled to 33° to 34°C.

After weaning from CPB, protamine was administered 0.8 to 1 mg/100 IU of the initial heparin dose. After CPB, mean arterial pressure of 60 to 80 mm Hg was targeted. Hypotension after CPB was treated first by fluid loading (Ringer's acetate; albumin, 4%) to achieve a pulmonary capillary wedge pressure of 12 to 15 mm Hg. Thereafter, norepinephrine infusion (0.01 to 0.1 $\mu g \cdot kg^{-1} \cdot min^{-1}$) was commenced if needed. After CPB, a cardiac index of more than 2.0 $L \cdot min^{-1} \cdot m^{-2}$ was maintained by preload optimization (pulmonary capillary wedge pressure of 12 to 15 mm Hg) as well as epinephrine (0.02 to 0.2 $\mu g \cdot kg^{-1} \cdot min^{-1}$) and milrinone infusion (0.5 $\mu g \cdot kg^{-1} \cdot min^{-1}$) if needed. Hemoglobin target level was less than 70 g/L during CPB and less than 80 g/L off-pump.

In addition to arterial samples, blood samples were obtained from the coronary sinus catheter. Correct placement of the catheter was verified with transesophageal ultrasound. Comparisons of partial pressure of oxygen (Po_2) and oxygen saturation (So_2) between simultaneously taken blood samples from the coronary sinus and pulmonary artery (ie, the mixed venous sample) were made in parallel. Lower Po_2 and lower So_2 in the coronary sinus than in the pulmonary artery was assumed to indicate correct placement of the coronary sinus cannula.

Blood samples for research were drawn at four time points: (1) before induction of anesthesia, "preoperatively"; (2) immediately before ischemia (ie, immediately before aortic cross-clamping), "preischemia"; (3) immediately before reperfusion (ie, immediately before aortic declamping, "prereperfusion"; and (4) 5 minutes after reperfusion, "reperfusion." At all time points, 15 mL of arterial blood was drawn. At time point 1, the sample was taken from the peripheral arterial cannula. At time points 2 to 4, blood was drawn from the arterial line of the CPB. At time points 2 and 4, parallel blood samples of 15 mL were drawn from the coronary sinus into pyrogen–free syringes (BD Plastipak, Madrid, Spain).

Samples were immediately divided into 2 vacuum tubes containing ethylenediaminetetraacetic acid (BD Vacutainer, Plymouth, United Kingdom) and 1 tube containing sodium citrate (BD Vacutainer). One ethylenediaminetetraacetic acid tube was used for automated leucocyte differential

count (Sysmex XE-2100; Sysmex Europe GmbH, Norderstedt, Germany). The rest of the tubes were transferred to ice-water bath, and plasma was separated within 20 minutes by centrifugation at 2,000*g* at 4°C. Plasma was stored in aliquots at –80°C.

Commercial enzyme-linked immunosorbent assay kits were used for measurements of HBP (Axis-Shiled Diagnostics, Dundee, United Kingdom), LF (Hycult Biotech, Uden, The Netherlands), MPO (BioLegend, San Diego, CA), and heart-type fatty acid binding protein (hFABP; Hycult Biotech, Uden, The Netherlands). Because hFABP is a rapid and sensitive biomarker of cardiomyocyte injury, it was used as a positive control biomarker for detection of transcoronary plasma concentration differences of an indicator [20]. Plasma aliquots that were not thawed previously were used for enzyme-linked immunosorbent assay measurements. Measurements of LF were originally conducted for another so far unpublished study, and the transcoronary difference of LF was not available at the "preischemia" assessment. In addition, troponin T was measured at the clinical laboratory of the hospital exactly at 22 hours and 48 hours after aortic declamping.

Data were analyzed with SPSS 25 software (IBM, Armonk, NY). The study was observational by nature. Because there was no intervention, power analysis for the size of a treatment group was not applicable. A nonparametric approach was used because of the small patient number. The Friedman test was used for testing differences as a function of time. Post hoc, differences between every pair of two consecutive time points (preoperative versus preischemia, preischemia versus prereperfusion, and prereperfusion versus postreperfusion) were tested using the Wilcoxon signed rank test with Bonferroni correction for three comparisons. Likewise, comparison of fold-increase between HBP, LF, and MPO at a same time point was undertaken with the Friedman test and Wilcoxon signed rank test as a post hoc test with Bonferroni correction for three comparisons (HBP versus LF, HBP versus MPO, and LF versus MPO). The Wilcoxon signed rank test was also used for comparison of transcoronary differences. The Spearman test was used for bivariate correlations. After Bonferroni correction resulting from three comparisons, p values of less than 0.017 were considered statistically significant. Otherwise, p values of less than 0.05 were considered significant. Data are expressed as median and interquartile range (IQR) or depicted as box plots.

Results

The study group consisted of 15 men and 15 women. The age of the patients was 66 years (IQR, 61 to 73 years), the CPB time was 101 minutes (IQR, 85 to 114 minutes), and aortic cross-clamping time was 70 minutes (IQR, 58 to 79 minutes).

Verification of the Placement of the Coronary Sinus Catheter

In the verification of correct placement of the coronary sinus catheter, Po_2 and So_2 were higher in the coronary sinus sample than in the simultaneously taken mixed venous sample in 1 patient before CPB (the sample was deleted from all analyses). The catheter was readjusted and the following samples proved valid Po_2 and So_2 measurements. In all other patients, Po_2 and So_2 were lower in the coronary sinus sample than in the simultaneously taken mixed venous sample before CPB and also after reperfusion (data not shown). As a positive technical control biomarker for the measurement of transcoronary plasma concentration differences, hFABP was significantly higher in the coronary sinus samples than in the simultaneously taken arterial samples before CPB (artery: 9.8 ng/mL [IQR, 6.0 to 17.7 ng/mL]; coronary sinus: 11.2 ng/mL [IQR, 6.2 to 18.3 ng/mL]; difference: 0.4 ng/mL [IQR, –0.04 to 3.5 ng/mL]; $p = 0.002$) and after reperfusion (artery: 36.7 ng/mL [IQR, 23.9 to 59.2 ng/mL]; coronary sinus: 46.1 ng/mL [IQR, 28.8 to 90.5 ng/mL]; difference: 6.9 ng/mL [IQR, 3.0 to 25.8 ng/mL]; $p < 0.001$).

Changes in the Arterial Samples as a Function of Time

Plasma concentrations of HBP, LF, and MPO, as well as neutrophil count in arterial samples as a function of time are presented in Table 1. All of these indicators increased significantly during the study period ($p < 0.001$ for all indicators). In HBP, LF, and MPO concentrations as well as in neutrophil count, there was a significant difference (all $p < 0.01$) in every pair of two consecutive time points (preoperative versus preischemia, preischemia versus prereperfusion, and prereperfusion versus postreperfusion). There were significant increases also in monocyte and lymphocyte counts in arterial samples during the study period (data not shown). For the relative comparison between HBP, LF, and MPO, granule protein levels at different time points are presented as fold-increase (ie, as multiples of respective baseline values in Fig 1). During the operation, HBP increased 39-fold, LF 16-fold, and MPO fourfold compared with the preoperative level.

Changes Across the Coronary Circulation

Before CPB, HBP was marginally but statistically significantly higher in the coronary sinus than in the simultaneously taken arterial sample (Fig 2). Transcoronary gradients were not observed before CPB for neutrophil (Fig 2), monocyte (data not shown), or lymphocyte counts (data not shown) or for MPO concentrations (Fig 2). After reperfusion, the neutrophil count was significantly lower in the coronary sinus than in the arterial sample, indicating a transcoronary neutrophil sequestration of -0.14×10^9/L (IQR, -0.28 to 0.01×10^9/L; $p = 0.001$, Fig 2). After reperfusion, there were also statistically significant entrapment of monocytes (transcoronary difference: -0.06×10^9/L; IQR, -0.11 to 0.02×10^9/L; $p = 0.013$) and lymphocytes (transcoronary difference: -0.020×10^9/L; IQR, -0.065 to -0.005×10^9/L; $p = 0.021$). After reperfusion, a positive transcoronary HBP concentration gradient was observed, indicating transcoronary release of HBP. This transcoronary HBP concentration difference did not correlate with transcoronary neutrophil difference, ischemia time, or troponin T at 22 hours or 48 hours after cardiac reperfusion. Transcoronary concentration

Table 1. Arterial Values of the Indicators During the Study Period[a]

Variable	Preoperative Median (IQR)	Preischemia Median (IQR)	Prereperfusion Median (IQR)	Postreperfusion Median (IQR)
HBP, ng/mL	4.1 (3.6–5.3)	41.0 (34.4–54.9)	132.7 (96.1–198.9)	150.0 (108.2–188.6)
Lactoferin, ng/mL	61.7 (48.4–132.4)	402.8 (310.6–541.1)	975.0 (598.0–1,275.3)	1,176.5 (653.2–1,377.0)
MPO, ng/mL	58.5 (42.4–75.5)	231.3 (142.2–257.2)	372.1 (273.9–426.2)	411.2 (287.2–465.8)
Neutrophils, $\times 10^9$/L	4.1 (3.1–4.6)	2.4 (1.5–3.6)	5.5 (3.6–8.9)	6.0 (4.4–8.7)

[a] All variables increased significantly during the study period (all $p < 0.001$).

HBP = heparin binding protein; IQR = interquartile range; MPO = myeloperoxidase.

gradients of neither LF nor MPO were observed before CPB or after reperfusion (Fig 2).

Comment

The present results have a threefold message. First, a substantial increase in plasma concentrations of HBP occurs during CPB. Second, in addition to systemic increase, HBP is also released locally in the reperfused coronary circulation, concomitantly with coronary neutrophil adhesion. Third, as a biomarker of intravascular neutrophil activation, HBP is superior to granule proteins of specific granules (LF) or azurophilic granules (MPO).

The median level of HBP is approximately 30 ng/mL in septic patients without organ dysfunction and approximately 50 ng/mL in patients with organ dysfunction [3, 5]. In our patients undergoing cardiac operations, a median HBP level of 41 ng/mL was already reached before CPB, and a median level of 150 ng/mL was observed after reperfusion. In septic patients, a plasma HBP concentration higher than 15 ng/mL predicts development of organ dysfunction and is associated with fourfold increase in the risk of death [3]. Because the clinical scenario of sepsis is different from that of CPB, a direct comparison of septic and cardiac surgical patients cannot be done.

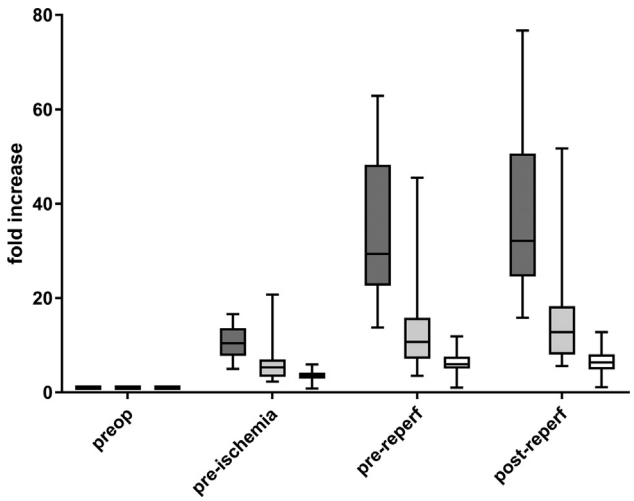
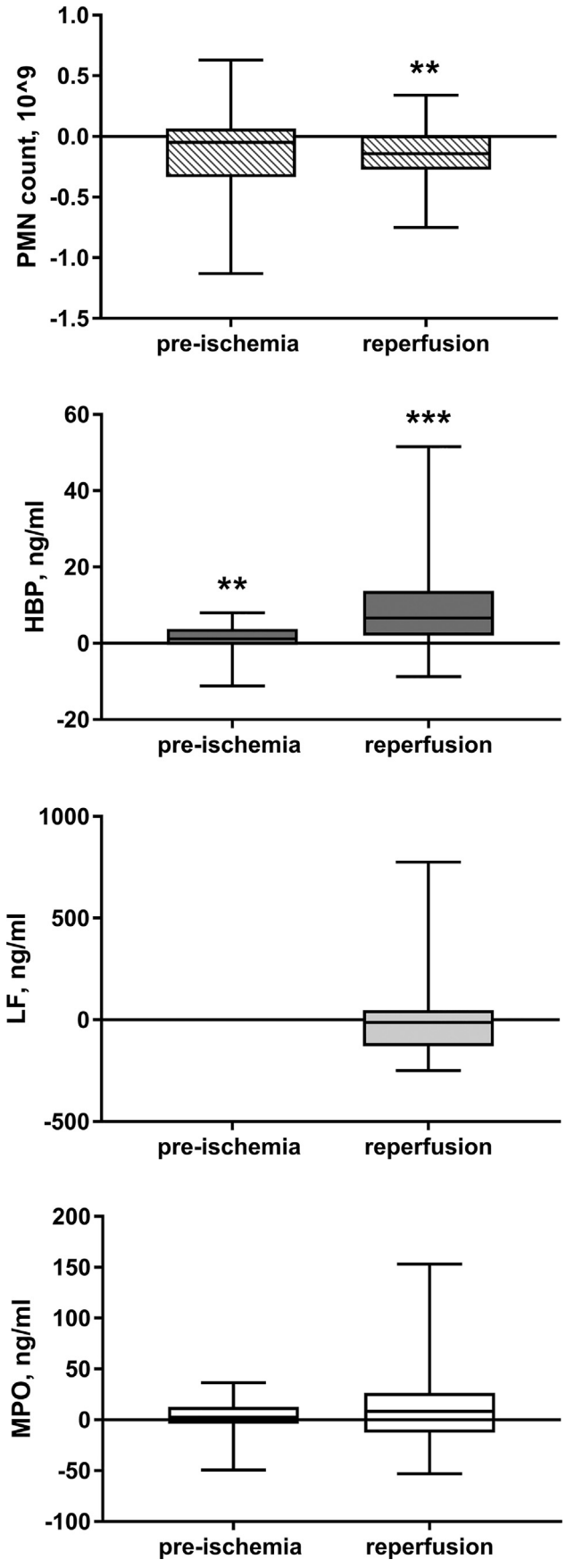


Fig 1. Plasma levels of heparin binding protein (HBP, dark grey boxes), lactoferrin (LF, light grey boxes), and myeloperoxidase (MPO, white boxes) in arterial samples during the study period are presented as fold-increase (ie, as multiples of respective baseline values). All indicators increased significantly (all $p < 0.001$). HBP, LF, and MPO all differed from each other before cardiopulmonary bypass, before reperfusion, and after reperfusion (all $p < 0.001$). The horizontal line in the middle of each box indicates the median, the top and bottom borders of the box mark the 75th and 25th percentiles, respectively, and the whiskers mark minimum and maximum of all the data. (post-reperf = postreperfusion; preop = preoperative; pre-reperf = prereperfusion.)

Still, substantially high levels of HBP were observed during CPB. HBP induces vascular leak in experimental conditions [11, 14]. In septic patients, HBP is correlated with the degree of fluid overload and hypoxemia [14]. Plasma obtained from CPB patients mediates endothelial hyperpermeability in vitro [16]. Clinical significance of HBP cannot be judged in the present observational study with a small patient number. Nevertheless, it is tempting to speculate that HBP may be one candidate for a humoral mediator of hyperpermeability during CPB [15, 16].

Numerous experimental studies have shown the pathophysiological significance of activated neutrophils in experimental cardiac ischemia-reperfusion injury [17]. Although intracoronary entrapment of neutrophils during reperfusion in cardiac surgery was observed long ago, conclusive evidence of transcoronary neutrophil activation has been difficult to obtain [18, 21]. Because lymphocytes and monocytes are also sequestrated in the coronary circulation, one may ask whether coronary leukocyte entrapment in clinical heart surgery is only a passive phenomenon. During cardiac reperfusion at 5 minutes after aortic declamping, we observed statistically highly significant release of HBP in the coronary circulation. A median transcoronary HBP concentration difference of 6.6 ng/mL was observed, and the highest measured difference was 51.6 ng/mL. Because we took simultaneous samples of blood entering and leaving the coronary circulation, the transcoronary concentration difference measured momentous production of HBP. The cumulative production was likely even more excessive. Furthermore, because HBP is bound to the endothelial glycocalyx, the observed transcoronary concentration difference probably reflects only a spillover of all HBP released from the adhered neutrophils.

Taken together, compared with systemic HBP levels in cardiac surgical patients and especially in septic patients, intracoronary production of HBP can be regarded strong [3, 5]. Two conclusions can be drawn. First, intravascular neutrophil activation in the coronary circulation indeed occurs during cardiac reperfusion in clinical heart surgery.



Second, this neutrophil activation results in high local concentration of HBP in the coronary vascular bed. Again, it can be speculated that accumulating HBP may have significance in coronary endothelial cell injury and myocardial edema formation, occurring both in experimental and clinical cardiac surgery [22, 23]. Of note, coronary HPB release was accompanied with the coronary release of hFABP, an early and sensitive biomarker of myocardial injury [20].

Compared with the preoperative level, HBP increased up to 39-fold after aortic declamping. The corresponding increases in LF were 16-fold and in MPO were only fourfold. Approximately the same proportional differences in plasma concentrations of the 3 granule proteins were already observed at the beginning of CPB (ie, at a moderate level of neutrophil activation). Furthermore, although clear coronary release of HBP was observed after cardiac reperfusion, LF and MPO both failed to detect intracoronary neutrophil activation. Of note, a marginal but statistically significant transcoronary increase of HBP was observed even immediately before aortic cross-clamping. Heparinization and initiation of CPB probably result in increased circulating HBP concentrations at this early phase of the operation. However, concomitant marginal release of also hFABP was observed. Thus, these two preischemic findings may also reflect subtle myocardial and endothelial injury caused by cannulation and other surgical manipulation of the heart before CPB.

These findings further underscore the sensitivity of HBP as a biomarker. The differences between neutrophil granule protein concentrations reflect the timing and sequence of neutrophil degranulation during physiological neutrophil functions. On one hand, β_2-integrin signaling triggers the release of HBP-containing secretory vesicles upon neutrophil adhesion to the endothelium (ie, in the intravascular space) [11]. On the other hand, azurophilic granules (MPO) are important in the formation of a phagosome for killing of microbes in the tissues [2]. Specific granules (LF) are somewhere in between. They take part in phagosome formation but they also contain β_2-integrins that are needed in endothelial adhesion [2]. The fact that secretory vesicles are meant to be released intravascularly probably makes HBP a superior neutrophil biomarker in plasma.

Neutrophilic granule proteins of only either specific or azurophilic granules have been measured in cardiac surgery in the past. No clinical effect has been reached in these studies. In future studies, HBP as a biomarker may offer a different picture of the clinical significance of neutrophil activation in cardiac surgery.

*Fig 2. Transcoronary differences (ie, the difference between coronary sinus and artery) of neutrophil count and plasma concentrations of polymorphonuclear (PMN) cells, heparin binding protein (HBP), lactoferrin (LF, preischemia missing), and myeloperoxidase (MPO) before cardiopulmonary bypass and 5 minutes after aortic declamping. The horizontal line in the middle of each box indicates the median; the top and bottom borders of the box mark the 75th and 25th percentiles, respectively, and the whiskers mark minimum and maximum of all the data. **$p < 0.01$, coronary sinus versus artery; ***$p < 0.001$ coronary sinus versus artery.*

The chosen patient population is both a strength and a weakness of the study. Our focus was on the coronary reperfusion phenomenon, and thus, coronary sinus blood samples were obtained. We wanted to avoid the confounding effect of coronary artery disease. This reduced not only the number of patients suitable for the study but also ischemia times and thus variability of ischemic challenge. A small and homogenous patient population and fairly subtle ischemic insult may explain the fact that we did not find correlations between HBP and clinical indices (ie, troponin or aortic cross-clamping time). However, both the preischemic and postischemic coronary HBP release followed the pattern of the coronary hFABP release. Although substantial coronary release of HBP was observed in patients undergoing isolated aortic valve replacement, it is likely that coronary neutrophil activation would have been even stronger in patients with concomitant coronary pathology and longer ischemia times. Thus, the present patient population serves as a meaningful verification of reperfusion-induced coronary neutrophil activation in clinical heart surgery.

Correct placement of the coronary sinus catheter was rigorously verified with multiple methods (ie, transesophageal cardiac ultrasound as well as measuring P_{O_2}, S_{O_2}, and hFABP). Furthermore, artifact neutrophil activation in vitro was reduced to a minimum when blood samples for separation of plasma were immediately cooled on an ice-water bath, centrifuged at 4°C, and plasma separated within 20 minutes.

In conclusion, CPB induced a substantial increase in HBP plasma concentrations in the systemic circulation. Furthermore, HBP was also released in the reperfused coronary circulation at the time of coronary neutrophil adhesion and myocardial injury. Both experimental in vitro and in vivo evidence has accumulated for the key role of HBP in the pathophysiology of capillary leak [11, 14]. According to the present results, HBP is one candidate for a humoral factor mediating capillary leak in CPB [16]. In addition, acute kidney injury is a frequent complication of CPB. HBP predicts acute kidney injury in septic patients [8, 9]. HBP may perform well as a biomarker of organ dysfunction also in cardiac surgery. The clinical significance of HBP needs to be verified in a larger patient cohort.

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