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Assessment of xanthine oxidase in human lung and lung transplantation

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ABSTRACT: Oxygen free radical generation by xanthine oxidase (XO) is a possible mechanism in the injury following reperfusion of transplanted organs. This study was undertaken to investigate XO in human lung, and to investigate whether XO is released into the blood stream during the immediate postoperative period after lung transplantation.

XO activity was measured in healthy human lung tissue, and XO protein and the adenine nucleotide catabolic products hypoxanthine, xanthine and uric acid were analysed in the plasma samples collected during human heart-lung transplantation (n=4), double lung transplantation (n=2), and single lung transplantation (n=1). Neutrophil degranulation was assessed by plasma lactoferrin measurements.

The results indicated that XO activity (detection limit 5 pmol·min^{-1}·mg^{-1} protein) and protein (detection limit 5 ng·mg^{-1} protein) were undetectable in the lungs of five healthy individuals. Similarly, no XO protein could be found in the plasma samples from the right ventricle or left atrium during and after the transplantation in any of the cases. Plasma xanthine and hypoxanthine concentrations were elevated 2–10 fold immediately after the reperfusion of the transplant, indicating washout of high-energy phosphate degradation products from the ischaemic lung. Plasma uric acid decreased rather than increased immediately after the surgery and during the following 24 h. Lactoferrin was elevated during the surgery.

In conclusion, these results show that XO activity in human lung is low, it is not released into the blood stream during human heart-lung transplantation, and it is unlikely to contribute to postoperative complications in these patients.

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Studies in experimental animals and isolated organs have indicated that reactive oxygen metabolites play a central role in the development of ischaemia-reperfusion injury [1–3]. In human lung and heart-lung transplantations, postoperative problems in the function of the transplanted organs are common, and may prove fatal. Rejection does not account for the pathogenesis of early organ dysfunction, and thus ischaemia-reperfusion damage merits a closer analysis as another potential mechanism of injury.

The most likely sources of free radicals are xanthine oxidase (XO) [1] and activated neutrophils [4]. XO has been widely investigated in the context of superoxide and hydrogen peroxide generation during ischaemia-reperfusion and organ transplantation. Breakdown of high-energy nucleotides to hypoxanthine to provide substrate for XO, and conversion of the normally occurring xanthine dehydrogenase into the superoxide-producing oxidase form are the cornerstones of this hypothesis [1]. XO activity is high, especially in liver and intestine [4, 5], and evidence has been presented for its role in the development of ischaemic intestinal, hepatic, and renal damage [6–8]. It may also contribute to the development of lung and myocardial reperfusion injury after ischaemic episodes, although this conclusion is based on indirect evidence showing attenuation of the injury by XO inhibitors in experimental animals [9–11]. Xanthine oxidase-related tissue injury is not necessarily proportional to whole tissue enzyme activity, because it appears to be localized mainly in capillary endothelium [12]. Furthermore, the enzyme can be released into the circulation and cause injury far from the site where it is formed [13].

Recent evidence also implicates neutrophil-derived oxygen radicals as potential contributors to the development of tissue injury [14–16]. Neutrophil activation and adhesion to microvascular endothelial cells may significantly predispose the target tissue to ischaemia-reperfusion injury [4, 8, 17, 18]. Activation is accompanied by degranulation and release of myeloperoxidase and lactoferrin from neutrophils.

There is insufficient information on the localization of XO in human tissues and its significance in human diseases. No studies have been reported on XO in human lung transplant patients. In the present study, XO activity was measured in healthy human lung and in plasma of human heart-lung and lung transplant patients. Furthermore, nucleotide degradation products, which reflect

substrate provision for XO, were measured in the same patients during different time-points of the surgery. Lactoferrin was analysed in plasma to evaluate degranulation of neutrophils, a potential mechanism of reperfusion damage.

Given that free radicals may be involved in the postoperative complications of lung transplantation, XO, its reaction metabolites, such as uric acid, as well as lactoferrin (as a marker of neutrophil degranulation), were assessed during the immediate postoperative period of these patients.

Methods

Four patients underwent heart-lung transplantation: two for primary pulmonary hypertension; one for chronic obstructive lung disease and right-sided heart failure; and one for ventricular septal defect and Eisenmenger's syndrome. In addition, one patient with emphysema and another with idiopathic primary pulmonary hypertension (PPH) underwent sequential double lung transplantation, and one patient with emphysema received a single lung transplant. The mean age of the patients was 45 yrs (range 28–53 yrs). The transplantations were conducted at Helsinki University Central Hospital, Department of Thoracic and Cardiovascular Surgery.

All organ donors received prostaglandin E_1 (Prostivas; Upjohn, Kalamazoo, MI, USA), 30–150 ng·kg^{-1}·min^{-1}, until systemic arterial pressure decreased by 30%, whereafter the heart was protected with 500–1,000 mL of cold cardioplegia (Plegisol; Abbott, Chicago, IL, USA). The lungs were perfused with cold modified Euro-Collins solution (60 mL·kg^{-1} for 4 min) (Fresenius AG, Germany), and stored in 4°C Ringer acetate solution until transplanted. The median ischaemia time in heart-lung transplantations was 182±33 min (mean±SD) (range 155–225 min), and in the lung transplantations 215±46 min (range 177–277 min).

Patients requiring heart-lung transplantation and cardiopulmonary bypass were anaesthesized with high-dose fentanyl and diazepam, and relaxed with pancuronium. Patients undergoing single and double lung transplantations were anaesthetized with thiopentone and low-dose fentanyl, and relaxed with pancuronium. Anaesthesia was maintained with fentanyl and isoflurane. One important difference between the operations was the cardiopulmonary bypass during the heart-lung surgery, whereas in lung transplantation no extracorporeal perfusion was used.

Immunosuppression after the induction and during the operation included intravenous antithymocyte globulin (3–5 mg·kg^{-1}), intravenous methylprednisolone (250 mg before antithymocyte globulin, 500 mg before reperfusion, and 250 mg at the end of the surgery), and intravenous azathioprine (1–3 mg·kg^{-1}). Cyclosporine was started 24–48 h after the operation. No other investigational drugs were used. Intraoperative intravenous fluid and electrolyte administration, as well as diuretics and blood transfusions, were prescribed on clinical indications. Two patients (Nos. 3 and 4) died in the immediate postoperative period. Patient No. 3 died 12 h after the transplantation of acute myocardial infarction and pulmonary oedema. Patient No. 4 died after 2 weeks of invasive Aspergillus infection.

The study protocol was approved by the Ethics Committee of the hospital (Helsinki University Central Hospital, Dept of Thoracic and Cardiovascular Surgery), and informed consent was obtained from the patients.

Blood samples

During the heart-lung transplantation, blood samples were collected within 5 min after the induction, immediately after opening of the chest cavity, 5 min before reperfusion, 5, 15, 30 and 60 min, and in some cases also 5, 7, 12 and 24 h after reperfusion. During the first hour after reperfusion, samples were collected *via* direct puncture from the right ventricle and left atrium, and additional samples were also collected from the central venous catheter in the superior vena cava. The 5–24 h samples were obtained from the radial artery. In the two sequential double lung transplantations, samples were collected from the radial artery before the recirculation to the first lung, after 10 min of recirculation to the first lung, before recirculation to the second lung, and after 10 min of recirculation to the second lung. Centrifuged plasma samples were frozen at -70°C until analysis.

Assay of lung XO-activity.

Lung tissue for XO activity and XO protein measurements was obtained from double lung donors in connection with single lung transplantations or from healthy lung tissue during the surgery of lung tumours. Lung tissue was placed immediately into liquid nitrogen and stored at -70°C. For XO assay, the tissue was homogenized in 50 mM phosphate buffer (pH 7.8) containing 0.5 mM dithiothreitol, 0.5 mg·L^{-1} leupeptin, and 0.2 mM phenylmethylsulphonylfluoride. The homogenate was centrifuged (27,000×g for 30 min) at 4°C, and possible endogenous inhibitors were removed by gel filtration (PD10 column; Pharmacia, Uppsala, Sweden). XO was analysed using ^{14}C-xanthine (Amersham, Bucks, UK) as substrate, and the uric acid produced was measured by liquid scintillation after high performance liquid chromatography (HPLC) separation [19]. Protein was measured using the micromethod of Bio-Rad Chemicals (Hercules, CA, USA).

Xanthine oxidase protein was assayed in tissue homogenates and plasma with enzyme-linked immunosorbent assay (ELISA). Rabbit antibodies in the anti-XO antiserum, produced in our laboratory [20], were isolated by affinity chromatography on protein A Sepharose (Pharmacia, Sweden), and used at a concentration of 50 µg·mL^{-1} of 50 mM sodium carbonate buffer, pH 9.5, to coat microtitre plates (Immunoplate I; Nunc, Roskilde, Denmark) at 100 µL·well^{-1}. The plates were incubated overnight at 4°C and washed twice with 20 mM Tris-500 mM NaCl, (TBS) pH 7.5. Residual binding sites were blocked with 100 µL·well^{-1} of 2% bovine serum albumin (BSA) in TBS for 1 h at room temperature, and then washed once with 0.05% Tween 20 in TBS (TTBS). Plates coated with 100 µL·well^{-1} of 2% BSA in TBS were used as controls for the nonspecific binding [20].

Dilutions of human XO, purified from human milk in our laboratory [20], in 1% BSA-TTBS were used as standards covering the range 1–50 ng·mL^{-1}. Standards and samples diluted 1:5 or more were added, in a volume of 100 µL, in triplicate to the plates. Diluting buffer

was used as a blank. The plates were incubated overnight at room temperature, washed three times with 200 µL TTBS·well^{-1} after which 100 µL of the affinity purified anti-XO conjugated with alkaline phosphatase (Sigma, St. Louis, MO, USA) [21] was added to the wells, at a dilution of 1:300 in 1% BSA-TTBS. The plates were incubated for 4 h at room temperature and then washed four times. Fresh substrate (2 g of paranitrophenylphosphate) in 1 L of 100 mM diethanol-100 mM $MgCl_2$, pH 9.9, was added (100 µL·well^{-1}). After incubation (37°C for 45 min), the absorbances were recorded at 405 nm.

Xanthine, hypoxanthine and uric acid were measured in plasma using HPLC, as described previously [19].

Lactoferrin was assayed in plasma using ELISA in principle like the XO ELISA. Antilactoferrin antibodies were isolated from antiserum to human lactoferrin (Dako, Glostrup, Denmark) by affinity chromatography, using a column of lactoferrin-Sepharose. A portion of the purified antibodies (5 µg·mL^{-1}) was used for coating of the microtitre plates. Another portion of the purified antibodies was labelled with alkaline phosphatase and used for detection of the bound lactoferrin to the coated plates.

Statistical analysis

When indicated, data are expressed as mean±SD. Statistical comparison was performed using variance analysis and Scheffe's *post hoc* test. A p-value less than 0.05 was considered to be significant.

Results

Xanthine oxidase activity and XO protein were analysed in five healthy lung samples. In each case, the levels were undetectable (detection limit for XO by activity assay 5 pmol·min·mg^{-1} protein, and by ELISA 5 ng·mg^{-1} homogenate protein). In addition, no XO protein could be detected in the plasma samples from the right ventricular or left atrial effluents, or from the radial artery in heart-lung or lung transplant patients in any of the cases (detection limit 5 ng·mL^{-1} plasma). As discussed below, we have found significant XO levels in human liver and intestine, the levels being 146 ng·mg^{-1} tissue protein and 556 ng·mg^{-1} respectively [20].The findings suggest that significant amounts of XO are not present in transplanted human lungs, nor is the enzyme released during human heart or lung transplantation.

Catabolic products of purine nucleotides were analysed in the plasma samples. Hypoxanthine levels after heart-lung transplantation showed considerable variation, possibly due to different preservation and ischaemic times before the reperfusion. However, there was a consistent 2–10 fold increase after reperfusion, with a maximum at 10–20 min after reperfusion. Subsequently, hypoxanthine concentrations returned towards the baseline values (fig. 1, patients Nos. 1–4). Only a marginal increase in the plasma hypoxanthine levels was observed in the patient undergoing single lung transplantation (fig 1, patient No. 5). In sequential lung transplantations (patients Nos. 6 and 7), two hypoxanthine peaks were observed, the first appearing within the first 10 min after the opening of the first graft, and the second immediately after the opening of the second graft (fig. 2). Plasma xanthine

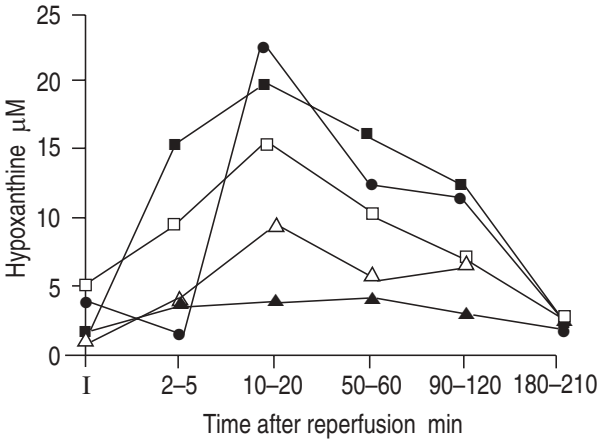

Fig. 1. – Individual plasma hypoxanthine concentrations in the four heart-lung transplant patients (Nos. 1–4) and in one single lung transplant patient (No. 5) at different time-points of the surgery. I: Induction. —●—: No. 1; —□—: No. 2; —■—: No. 3; —△—: No. 4; —▲—: No. 5.

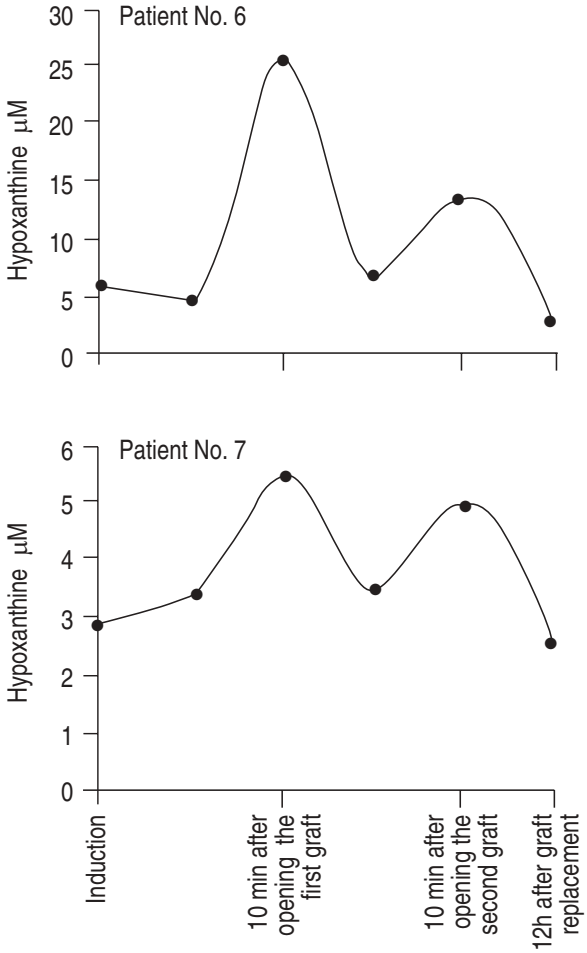

Fig. 2. – Individual plasma hypoxanthine concentrations in the two sequential double-lung transplant patients (Nos. 6 and 7) at different time-points of the surgery.

levels were elevated after the reperfusion, but to a smaller degree than hypoxanthine (not shown). Elevated hypoxanthine and xanthine levels merely indicate washout of high-energy phosphate degradation products from the ischaemic tissue, which does not indicate XO activity. Plasma uric acid concentrations, representing a product

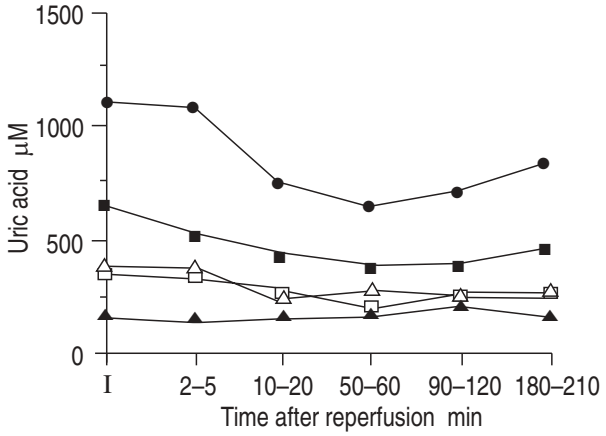

Fig. 3. – Individual plasma uric acid concentrations in the four heart-lung transplant patients (Nos. 1–4) and one single lung transplant patient (No. 5) at different time-points of the surgery. I: Induction. —●—: No. 1; —❑—: No. 2; —■—: No. 3; —Δ—: No. 4; —▲—: No. 5.

of XO, showed a wide range, but there was a tendency towards a decrease rather than an increase after the heart-lung transplantation (fig. 3). Hypoxanthine, xanthine and uric acid concentrations did not differ significantly in samples collected from the radial artery, right ventricle, or left atrium.

Plasma lactoferrin concentration was used as a marker of granulocyte degranulation. The levels increased during the surgery, as shown in figure 4. In contrast to hypoxanthine, plasma lactoferrin was already significantly elevated after the beginning of surgery before the reperfusion, and the levels were further increased after reperfusion; the values being: 0.22±.14 $\mu g \cdot mL^{-1}$ after the induction; 1.4±0.8 $\mu g \cdot mL^{-1}$ 5 min before reperfusion; and 3.4±1.1 $\mu g \cdot mL^{-1}$ within the first 30 min after reperfusion, respectively. In one heart-lung transplant patient, blood leucocyte counts in the right ventricular and left atrial samples collected during the first 12 h did not differ, the values being $19.1 \pm 6.3 \times 10^6$ and $20.4 \pm 4.8 \times 10^6$, respectively.

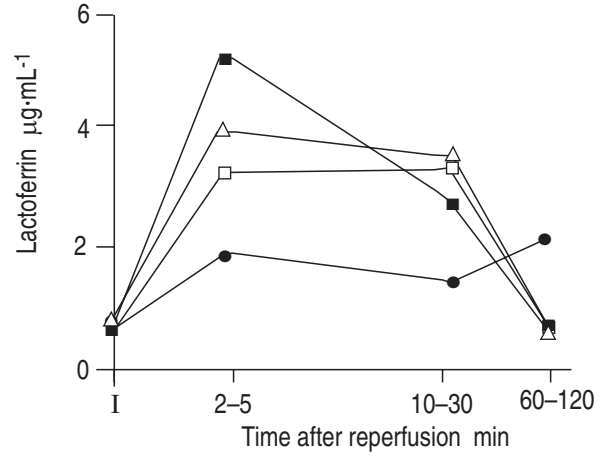

Fig. 4. – Individual plasma lactoferrin concentrations, as a marker of neutrophil degranulation in the four heart-lung transplant patients (Nos. 1–4) at different time-points of the surgery. I: Induction. —●—: No. 1; —❑—: No. 2; —■—: No. 3; —Δ—: No. 4.

Discussion

These results show that xanthine oxidase activity in human lung is low, it is not released into the blood stream during lung transplantation, and may not explain post-operative complications of transplanted lung.

Ischaemic cell and tissue injury results in depletion of adenosine triphosphate (ATP) and increase of purine catabolic products [1, 19, 22, 23], mainly hypoxanthine, xanthine and uric acid. Hypoxanthine efflux has recently been shown to correlate with the ischaemic time of the cardiac transplant, and has been suggested to be a sensitive and objective biochemical indicator of graft preservation and immediate function [24]. In agreement with previous studies on human myocardial ischaemia and cardiac allografts [24, 25], the present patients had elevated plasma hypoxanthine levels after graft reperfusion. However, plasma uric acid levels, which might indicate XO activation, were unchanged.

The role of XO-derived free radicals was originally proposed in ischaemia-reperfusion injury of the intestine, but the enzyme has since been implicated in similar damage to kidney, liver, heart and lung [4, 13]. Most of these studies have been conducted in experimental animals and/or perfused organs [6, 9, 10, 13, 15, 26], and, thus, the data may not be valid for human tissues, because of large differences in xanthine oxidase activity between species and also between organs in any given species [5]. For instance, XO activity is relatively high in bovine and rabbit lungs, and bovine capillary endothelium, whereas it is very low in human lung [5, 12]. Using this same method, we have recently measured significant XO activity and immunoreactive protein in human liver and intestine, the protein levels being 146 $ng \cdot mg^{-1}$ tissue protein and 556 $ng \cdot mg^{-1}$, respectively [20]. In agreement with previous data, we could not find XO in human lung. This does not rule out significant enzyme activity in a subpopulation of lung cells, *e.g.* capillary endothelium [12], but either the activity per cell or the number of such cells must be low.

XO may contribute to the development of cell and tissue injury in organ transplant patients, even if its activity in the target tissue is low. The enzyme can be released into the circulation and adhere to the microvasculature far from the site where it has been synthesized. Isolated perfused rat liver [13], as well as liver from a rat in haemorrhagic shock [26], released large amounts of xanthine oxidase into the circulation upon reoxygenation. We have measured significant XO activity and protein in plasma samples after reperfusion of transplanted human liver (unpublished). However, we were not able to detect XO activity or protein in the circulating plasma of the present patients after heart-lung or lung transplantation in any of the cases. Although the present material is small, all results obtained were very consistent, *i.e.* plasma XO levels were below the detection limit in every patient. Because the measurements were conducted from samples collected from both sides of the heart, and because the method for XO assay is very sensitive, significant XO release into the blood stream or adherence into the lung is not likely. The results suggest that if free radicals play a role in complications after human lung transplantation, they are probably derived from a source other than XO activation and/or release.

Although neutrophils are normally present in only small numbers in lung interstitium and alveolar spaces, a large circulating pool of neutrophils is available immediately after the reopening of the circulation. Activated neutrophils can cause lung injury by several mechanisms, the most important being release of reactive oxygen metabolites and myeloperoxidase. Possible contributing factors include neutrophil activation by various cytokines [27] and adhesion of neutrophils to the endothelium [8, 17, 28]. Lung neutrophil sequestration [29] and injury [30] have been demonstrated after intestinal ischaemia-reperfusion in experimental animals. Furthermore, plasma myeloperoxidase activity is significantly increased in the early phase after recirculation of the transplanted human kidney [31]. No studies on the relative role of XO and neutrophil activation in the ischaemia-reperfusion injury of the human lung are available. The present study showed a significant elevation of plasma lactoferrin levels in heart-lung transplant recipients immediately after reperfusion. Lactoferrin reflects neutrophil degranulation [32], which accompanies activation but is not directly correlated with neutrophil oxidant release.

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