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Rapid Communication

Ischemia–Reperfusion in Humans

Appearance of Xanthine Oxidase Activity

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We evaluated effluent blood from extremities of human patients undergoing reconstructive surgical treatment, which is routinely accompanied by upper-extremity exsanguination and application of a tourniquet, resulting in total interruption of arterial blood flow to one upper extremity. After tourniquet release (reperfusion), there were immediate increases in the plasma levels of xanthine oxidase activity, uric acid, and histamine in the ipsilateral limb and much smaller increases, if any, in levels of the same materials in plasma obtained from the contralateral extremity. There was no detectable xanthine dehydrogenase activity in plasma from either limb. Plasma also contained evidence of products consistent with the formation of oxygen-derived free radicals, namely, the appearance predominantly in the reperfused limb of hemoglobin and fluorescent compounds. These data indicate for the first time in humans that ischemia–reperfusion events are associated with the appearance of xanthine oxidase activity and its products in the plasma effluent. (Am J Pathol 1990, 136:491–495)

Reperfusion of tissues after a period of ischemia is well known to result in evidence of accentuated injury.[1] It has been speculated that the injury results from the generation of toxic oxygen products, which have been produced either from tissue enzymes or from activated phagocytic cells. The ability of allopurinol and superoxide dismutase to protect tissues from ischemia–reperfusion injury has been taken as evidence that generation of superoxide anion (O_2^-), perhaps from xanthine oxidase (XO), may be relevant to the pathogenesis of the tissue injury.[2] Recently, it has been suggested that generation of O_2^- results in formation of a chemotactic factor[3] that may be responsible for the recruitment of neutrophils into the involved tissue.[4] Indeed, previous neutrophil depletion of animals undergoing ischemia–reperfusion attenuates ischemia–reperfusion injury in the myocardium and small bowel.[5,6]

Although bovine and rat pulmonary artery endothelial cells contain xanthine oxidase, which during ischemia is increased due to the conversion of xanthine dehydrogenase (XD), catalytically active XO has not been detected in extracts of myocardium,[7] even though it has been shown that in a variety of human tissues there is immunochemical evidence for the presence of XO in vascular endothelial cells.[8,9] These observations have raised questions regarding the role of XO and its reaction products in ischemia–reperfusion events in humans.

Materials and Methods

Patients

Venous blood samples were obtained from 10 adult human patients undergoing elective surgical treatment of upper extremities. Centripedal application of elastic bands were applied to the extremity to be surgically treated to remove all remaining blood, followed by tourniquet application (varying from 60 to 120 minutes) to produce arterial compression and cessation of blood flow. At the end of this period the tourniquet was removed, restoring blood flow. Patients for study were selected at random and were treated in compliance with institutional proce-

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dures for the study of human subjects. No procedures were used that were not part of routine clinical practice for this type of surgical treatment. The mean age of the 10 patients was 49.09 ± 6.2 years (mean ± SEM). The mean tourniquet time was 90.2 ± 8.0 minutes. Surgical treatment included Dupuytren's contracture release, palmar tendolysis, ulnar nerve decompression, and other procedures. Tourniquet application was at a pressure approximately twice the systolic pressure sufficient to prevent bleeding in the surgical site. Reconstructive surgical procedures were performed in the absence of infection or other complicating factors. Before application and after release of the tourniquet, samples were obtained from venous effluent blood from the ipsilateral extremity (to which the tourniquet was applied) and from the contralateral extremity. No blood transfusions were used in treatment of patients. On the basis of clinical judgement, general or regional anesthesia was administered to these patients.

Materials

Unless otherwise stated, enzymes and reagents were purchased from Sigma Chemical Corp. (St. Louis, MO) and were of the highest purity available.

Sample Preparation and Storage

Using heparin-locked intravenous catheters, sequential venous blood samples were drawn from antecubital veins 10 minutes before placement of tourniquet and immediately after (within 20 seconds) as well as 3, 5, 10, 20, 30, 60, and 120 minutes after tourniquet release. Blood samples (2.5 ml) were obtained simultaneously from the ipsilateral (tourniquet-treated) and contralateral arms and were collected into tubes containing 10 molar (mmol/l) EDTA. Each sample was diluted immediately 1:1 (v/v) with a ice-cold medium consisting of 2.4 mmol/l potassium phosphate, 150 mmol/l sodium chloride, 10 mmol/l dithiothreitol (DTT), and 1 mmol/l phenylmethyl sulfonyl fluoride (PMSF) at a pH of 7.35. This was designed to prevent artifactual conversion of XD to XO. Plasma samples were obtained by centrifugation at 4 C within 10 minutes after sample collection. All samples were stored on ice and processed within 2 hours.

Xanthine Dehydrogenase/Xanthine Oxidase Activity

XD and XO activities were assayed spectrophotometrically by measurement of uric acid formation at 293 nm in the presence and absence of NAD^+ at 37 C, as described previously.[10] In some experiments O_2^- formation was directly measured on the basis of superoxide-inhibitable reduction of ferricytochrome c. In both systems of measurement 50 mmol/l xanthine was added to provide substrate for the enzyme.

Histamine Radioimmunoassay

Plasma histamine was determined by radioimmunoassay using a commercially available kit from AMAC Inc. (Westbrook, ME).

Hemoglobin Determination

Hemoglobin content in plasma samples was determined spectrophotometrically at 412 nm. Samples were diluted 1:50 (v/v) with buffer consisting of 2.4 mmol/l potassium phosphate, and 150 mmol/l sodium chloride, pH 7.35, before measurement.

Fluorescence Products

Fluorescence products in plasma were processed according the standard procedure in a fluorescent spectrofluorimeter, using excitation and absorbance wave lengths of 360 and 430 nm, respectively, as described previously.[11]

Uric Acid Determination

Plasma levels of uric acid were determined spectrophotometrically at 293 nm and expressed as mmol/l using a molar extinction coefficient of 7.59 cm^{-1} $mmol/l^{-1}$ for uric acid.[12] Proof that uric acid was measured was obtained by the addition of uricase, as described below.

Statistical Analysis

Data in various groups were expressed as mean ($\bar{X}$) ± standard error of the mean (SEM). To determine the significance of differences in samples obtained from contralateral (control) and ipsilateral (experimental) limbs, a paired *t*-test was used. Statistical significance was defined as $P < 0.05$.

Results

Appearance of Xanthine Oxidase Activity (XO) in Plasma

Levels of XO in plasma of the ipsilateral limb (to which the tourniquet was applied) and in the contralateral limb are shown in Figure 1A. The reference plasma was obtained from venous effluent blood before application of the tourniquet. With tourniquet release (reperfusion), there was a fivefold rise in XO activity in blood from the ipsilateral limb, the peak increase occurring at 3 minutes, followed by declining levels that were significantly elevated statistically above reference values for as long as 10 minutes. In blood from the contralateral limb, the statistically significant increase in XO activity was only found at 3 minutes and was two times more than the reference sample. At no time in either limb were there detectable levels of xanthine dehydrogenase (data not shown). The addition of 50 mmol/l allopurinol abolished the XO activity.

In an additional study it was possible to demonstrate that the ipsilateral (but not contralateral) plasma samples obtained 3 minutes after ischemia–reperfusion contained an allopurinol-inhibitable O_2^--generating activity as defined by SOD-inhibitable reduction of ferricytochrome c (data not shown). In companion studies involving plasma samples obtained from the venous drainage of the operated extremity of 5 patients, XO activities in the 0 and 3 minutes samples were 1.82 ± 0.35 and 7.97 ± 1.37 nmol uric acid formation/ml plasma/minute ($\bar{X}$ ± SEM), respectively. The addition of 50 mmol/l allopurinol caused activity in the two sets of samples to fall to 0.17 ± 0.10 and 1.19 ± 0.24 nmol uric acid formation/ml plasma/minute, respectively. Thus the activity measured in the plasma was highly susceptible to inhibition by allopurinol. Because XO reacts with available xanthine and hypoxanthine to form uric acid, plasma samples were evaluated for uric acid content by the spectrophotometric assays described above. As shown in Figure 1B, uric acid content was significantly elevated at 5 minutes in the plasma of the ipsilateral extremity, while no statistically significant rise was found in plasma from the contralateral extremity. Plasma samples were treated with 100 milliunits of uricase at 37 C for 20 minutes, resulting in a 73% reduction in the absorbance value, which reflects the presence of uric acid (data not shown).

In view of recent observations that histamine significantly enhances the catalytic activity of XO,[10] levels of histamine were measured in plasma from the ipsilateral and contralateral extremities. There were sharp increases (sixfold) in histamine content in plasma from the reperfused extremity during the first 30 minutes, but no statistically significant increases were found in plasma histamine in the contralateral limb (Figure 1C).

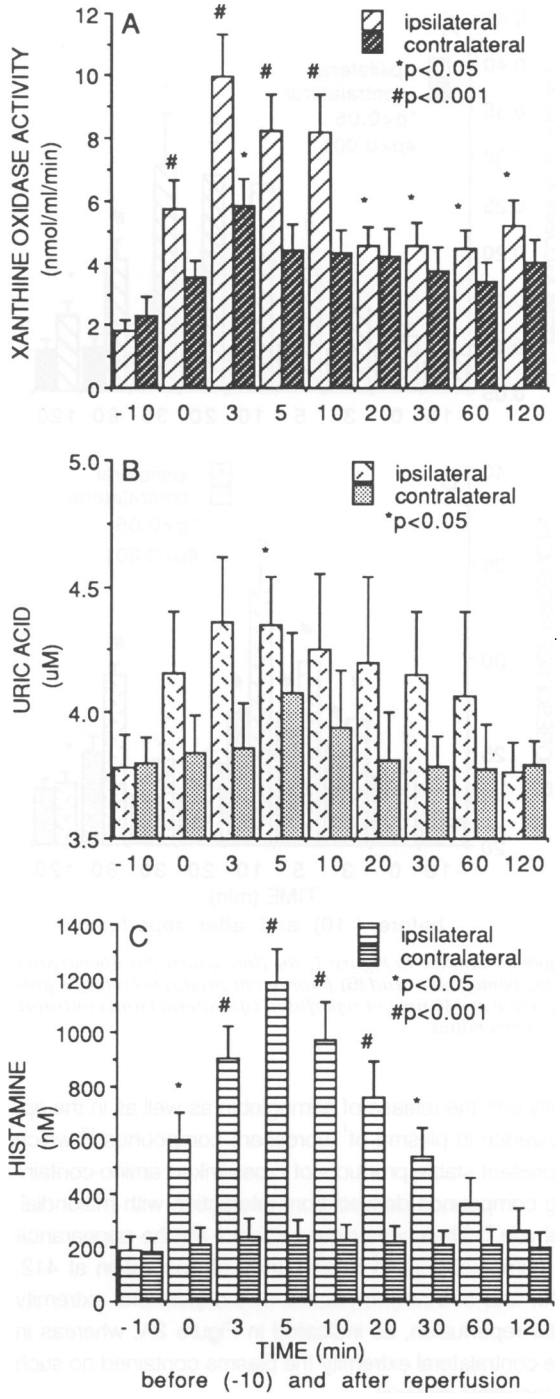

Figure 1. *Analysis of plasma from venous effluent blood of reperfused (ipsilateral) and control (contralateral) upper extremities in human patients undergoing reconstructive surgery. Shown are the time courses for plasma content of* (A) *XO,* (B) *uric acid, and* (C) *histamine.*

Presence of Hemoglobin and Fluorescent Products in Plasma

It has been shown that the intravascular generation of oxygen-derived free radicals results in damage of red blood

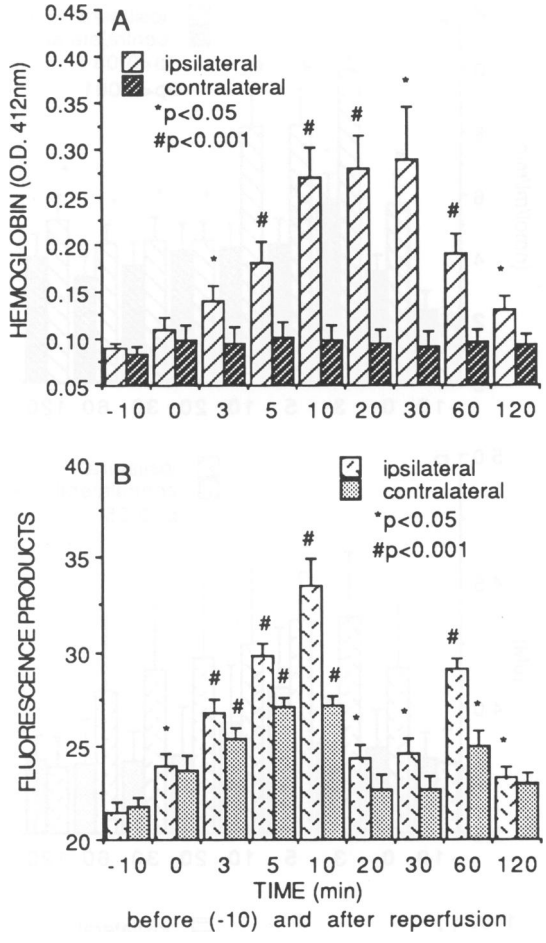

Figure 2. *Similar to Figure 1, the time course for appearance of* (A) *hemoglobin and* (B) *fluorescent products (relative fluorescence at 430 nm) in reperfused (ipsilateral) and contralateral extremities.*

cells with the release of hemoglobin as well as in the appearance in plasma of fluorescent compounds,[13] which represent stable products of cross-linked amino-containing compounds derived from interaction with malondialdehyde.[14] There was clear evidence for the appearance of hemoglobin (with Soret bands of absorption at 412, 540, and 578 nm) in plasma of the ipsilateral extremity after reperfusion, as indicated in Figure 2A, whereas in the contralateral extremity the plasma contained no such measurable material.

Fluorescent products were also found in the plasma of the ipsilateral extremity after reperfusion, as shown by the data in Figure 2B.

Discussion

These findings demonstrate for the first time that in ischemia–reperfusion events in humans, XO appears within the effluent blood of the involved tissues. The most probable source of the enzyme may be vascular endothelial cells that, having been altered by ischemia, release XO into the plasma during reoxygenation (reperfusion). Availability of substrate for XO through the breakdown of ATP may have occurred during the ischemic period, because it is now documented that cellular depletion of ATP (which would result in formation of adenine nucleotides, adenosine, inosine, hypoxanthine, and xanthine) occurs in such conditions.[15] The documented release of histamine during reperfusion (Figure 1C) could bring about enhancement of the catalytic activity of XO, as recently demonstrated both in XO of plasma and in intact endothelial cells,[10] making detection of XO possible. It is known that ischemia–reperfusion can bring about conversion within endothelial cells of XD to XO.[16] The combined results of all of these events could be endothelial cell damage, resulting in leakage of XO into the plasma of the reperfused extremity. While not shown by the data presented above, we have found a direct proportionality between the duration of ischemia (tourniquet application) and subsequent level of plasma XO, suggesting that the duration of ischemia and level of plasma XO may be directly linked. Although the data are incomplete, we have not, to date, found evidence of XO in the plasma of patients undergoing abdominal surgical interventions (data not shown). Also in burn patients who have undergone early eschar removal, we have failed to detect increases in plasma XO. If confirmed, these preliminary data would suggest that surgical trauma, *per se,* does not lead to a rise in plasma XO.

Although our data do not prove that ischemia–reperfusion events have resulted in the generation of oxygen-derived free radicals, the evidence supports this possibility. The appearance of fluorescent products with excitation/absorption wave lengths of 360/430 nm, respectively, is consistent with the generation of N-N′-iminopropenes derived from covalent cross linking of amino-containing compounds after reactivity with malondialdehyde, a product of lipid peroxidation and scission.[14] The presence of hemoglobin in plasma of the reperfused extremity could be due to limited destruction of red cells *via* generation of oxygen-derived free radicals by any of several mechanisms: the generation from xanthine by XO of O_2^-, which is cytotoxic for red cells,[17] the generation of toxic oxygen products from activated neutrophils,[18] or as a direct result of complement activation leading to reactive lysis mediated by the membrane-attack complex, C5b-9.[19] In this context, it has recently been suggested that formation of $HO^{\cdot}$ (either by xanthine oxidase or by activated neutrophils) can induce activation of the alternative complement pathway, resulting in either production of C5a (which could then activate neutrophils) or the generation of a C5 product causing red cell damage *via* reactive lysis.[20] Obviously, it is possible that the presence of

hemoglobin and fluorescent products could be due to mechanisms other than oxygen-derived free radical production. The definitive evidence would be whether the previous infusion of catalase and superoxide dismutase prevents the appearance of these products.

The issue of whether the plasma XO activity and its products contribute to injury in the context of human surgical interventions of the type described in this report can not be answered yet. Assuming the ability to define adequate endpoints, protective interventions using catalase and superoxide dismutase would be needed. It is our impression that in the upper extremity XO and its products may not contribute significantly to injury but in other organs, such as the myocardium, there may be greater tissue susceptibility and, perhaps, a larger amount of enzyme released after periods of ischemia.

The most definitive conclusion emanating from these studies is that, in humans with ischemia–reperfusion events occurring in an isolated extremity, XO activity appears in the plasma, although it is possible that in the limb the amount of XO is small and the availability of substrate is an even greater constraint. The recognition of appearance of XO in human plasma should pave the way for a series of studies to determine if similar responses occur in ischemia–reperfusion conditions involving tissues such as the myocardium, liver, and small bowel, and to determine the pathophysiologic significance of these events.

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