IMMUNOHISTOCHEMICAL EVIDENCE FOR XANTHINE OXIDOREDUCTASE IN HUMAN MYOCARDIUM

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Abstract

Xanthine oxidoreductase (XOR) could be an important source of reactive oxygen species (ROS) in the heart. The enzyme is clearly active in rat heart but its present in the human heart is controversial. This study was to detect XOR immunohistochemically in human myocardium using mouse raised monoclonal antibodies to purified human and bovine milk XOR. We assayed the presence of the enzyme in explanted human heart and compared to rat heart results. Using positive and negative controls, immunoactive XOR in heart was clearly present in the cardiomyocytes and in the smooth muscle cells of blood vessels in both species. The endothelial cells showed no staining in the smaller blood vessels. Other data suggest that ROS generating desulpho-type of XOR, present in rat heart, inactive with the substrate (hypo)xanthine is active using NADH as substrate, producing ROS. We speculate that this type of XOR plays a role in radical-induced cardiovascular disease.

***Key Words**: Explanted heart, Immunohistochemistry, Monoclonal Antibodies, Reactive Oxygen Species (ROS), and Xanthine oxidoreductase (XOR).*

Résumé

Xanthine oxydoréductase (XOR) peut bien être une source d'espèces réactives de l'oxygène (ERO) dans le cœur. Cette enzyme est clairement active dans le cœur du rat mais sa présence dans le cœur humain est controversable. Cette étude repose sur une détection immunohistochimique de l'enzyme dans le myocarde humain en utilisant des anticorps monoclonaux de souris anti-XOR Dans une étude comparative avec le cœur du rat, nous avons testé la présence de l'enzyme sur des biopsies du cœur humain. L'immunoactive XOR est clairement présente dans les cardiomyocytes et dans les cellules du muscle lisse des vaisseaux sanguins des deux espèces. Aucune coloration des cellules endothéliales des petits vaisseaux sanguins. D'autres résultats suggèrent que le générateur d'ERO type désulfo de la XOR du cœur du rat utilise le NADH pour produire les ERO. Nous spéculons que ce type de XOR pourrait jouer un rôle dans les maladies cardiovasculaires induites par les radicaux.

***Mots clés**: Anticorps Monoclonaux, Biopsie Cardiaque, Espèces Réactives de l'Oxygène (ERO), Immunohistochimie et Xanthine Oxydoréductase (XOR).*

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ملخص

الإنزيم المؤكسد المرجع للكسانثين (XOR) يمكن أن يكون مصدرا للجذور الحرة المأكسجة (ROS) في القلب. هذا الإنزيم ذو نشاطية واضحة في قلب الجرذ، بينما وجوده في قلب الإنسان موضع جدل. هذا البحث هو كشف عن الإنزيم في عضلة قلب (myocarde) الإنسان بطريقة مناعية نسيجية كيميائية. باستعمال، أجسام مضادة أحادية النسيلة ضد XOR تم اختبار الإنزيم على عينات نسيجية من قلب الإنسان. لغرض المقارنة شمل هذا البحث قلب الجرذ. باستعمال الشواهد (الموجب والسلبي)، الإنزيم النشط مناعيا موجود بوضوح في خلايا عضلات القلب (cardiomyocytes) وفي الخلايا العضلية الملساء للأوعية الدموية عند النوعين لكن غائبة في الخلايا البطانية. نتائج أخرى تقترح أن منتج الجذور الحرة نوع desulfo من XOR الموجود في قلب الجرذ يستعمل NADH كمادة تفاعل لإنتاج الجذور الحرة. يبدو أن هذا النوع يلعب دورا في أمراض القلب والشرايين الناتجة عن الجذور.

***الكلمات المفتاحية**: الإنزيم المؤكسد المرجع للكسانثين (XOR)، الجذور الحرة (ROS)، الأجسام المضادة أحادية النسيلة، الطريقة المناعية النسيجية الكيميائية.*

Xanthine oxidoreductase (XOR), commonly called xanthine oxidoreductase (XOR), commonly called xanthine oxidase, is an ase (XOR), commonly called xanthine oxidase, is an enzyme with a molecular weight of 300 KDa (homodimer of 150 KDa per subunit). The two identical subunits deriving from a single gene, contain molybdenum, iron-sulphur and FAD. The dehydrogenase from (EC 1.1.1.240) catalyses the rate-limiting steps in nucleic-acid degradation: hypoxanthine to xanthine, and xanthine to urate, concomitantly reducing NAD^+. Under pathological and physiological circumstances, rapid and cell-type specific conversion of the dehydrogenase from to the oxidase (EC 1.2.3.2.) takes place [1]. The oxidase from uses oxygen instead of NAD^+, and produces superoxide radicals ($O_2^{\cdot-}$) [2]. Free radicals have been implicated in cardiovascular disease, e.g. in tissue injury due to ischemia and reperfusion. A possible role of XOR as free-radical generator in the heart has made this enzyme a highly interesting candidate in the field of tissue damage. We detected a small production of urate during coronary angioplasty [3]. Others demonstrated xanthine production during cardiac operation [4]. As allopurinol was shown to inhibit the XOR activity and hence the formation of free radicals *in vitro* [5], several clinical trials indicated its usefulness for cardioprotection during heart surgery (for review, see [6]).

Cardiac XOR activity varies considerably among species. Mouse, rat and dog heart, e.g., contain high activity of XOR, but rabbit and pig heart it is almost nil [7-9]. Literature data on the presence of XOR in human heart are controversial. Several groups failed to detect appreciable XOR activity in human heart using spectrophotometric,

fluorometric, radio- or electrochemical techniques [10-12]. These findings contrast the results of Wajner and Harkness, who found high XOR activity in human myocardial post-mortem biopsies [13]. We calculated the XOR activity from the urate release after perfusion of hearts from different species with the substrate hypoxanthine; explanted human heart has a measurable but low activity of the enzyme [7]. Using histochemical methods various investigators showed XOR protein to be present in human myocardial tissue [14-17], whereas Kooij *et al*. could not find XOR activity in human post-mortem biopsies [18]. Finally, human atherosclerotic material contains a ferroxidase activity, attributable to XOR, was present in aneurysms and end arterectomies [19].

It has been known for years that vascular and cardiac tissues are rich sources of reactive oxygen species (ROS), including superoxide ($O_2^{\cdot -}$), hydrogen peroxide H_2O_2 and nitric oxide ($NO^{\cdot}$) (for review see [20,21]). At present, it seems that NAD(P)H oxidase is a major source of $O_2^{\cdot -}$ in vascular cells and monocytes [22]. XOR was shown to catalyse the reduction of nitrate to nitric oxide (NO) under anaerobic conditions, in the presence of either NADH [23] or xanthine as reducing substrates [24]. NO, a toxic and reactive gas, is a biological messenger and, over the last decade, has been the focus of intense research activity in its own right. Following early recognition of its vasodilatory action [25], NO has been found to play important regulatory roles not only in the cardiovasculature, but also in the central nervous system and to contribute to the microbicidal and tumouricidal activity of activated macrophages and neutrophils [26]. It is implicated in a wide range of physiological processes, including smooth muscle relaxation, inhibition of platelet aggregation, neurotransmission and immune regulation [26,27].

Also, the exact cellular localisation of XOR is still unclear. In human atrium XOR is reported to be localised in the microvasculature with background staining of the myocardium [16]. We previously determined by indirect immunofluorescence using confocal microscopy the subcellular localisation of XOR in human endothelial and epithelial cell lines and in primary cultures of human umbilical vein endothelial cells and fount that the enzyme was diffusely distributed throughout the cytoplasm but with higher intensity in the perinuclear region [28].

Hellsten-Westing found XOR to be mainly present in smooth-muscle cells of larger blood vessels, but also in endothelial cells of capillaries and small vessels in human cardiac muscle [14]. In contrast, Moriwaki *et al*. showed that XOR was only present in the endothelial cells of coronary arteries [17]. These controversial results could be due to the use of the different procedures to obtain pure XOR and antibody to this enzyme, besides different immunohistochemistry detection methods.

Our group has characterised XOR, purified from human milk [29,30]. We raised monoclonal antibodies to the enzyme purified from both human and bovine milk [31]. With these antibodies we localises XOR in fresh pieces of human ventricle. Myocytes, smooth-muscle cells and certain endothelial cell contained the xanthine-oxidase protein.

MATERIAL AND METHODS

Ethics

The investigation conformed with principals outlined in the declaration of Helsinki and the Guide for the Care and use of laboratory Animals (US National Institute of Health).

Tissue collection

Human hearts were obtained from transplantation patients with end- stage heart failure. The hearts were arrested with cold St. Thomas' hospital cardioplegic solution, excised and cooled on ice. Samples from the left ventricle were taken within 10 min.

Male Wistar rats (wag / Rig inbred, weighing 290-400g) were obtained from Harlan -CPB, Zeist, the Netherlands. Groups of animals were housed in makrolon cages on pre-sterilized wood chips. They received a commercial rat chow (Hope Farms AM II, Warden, The Netherlands) and tap water ad libitum. Anaesthesia took place with 0,7 ml pentobarbital, given i. p. With 0.1ml heparin. The hearts were rapidly excised, cold in saline (0°C) until contraction stopped, and thoroughly flushed with saline to get rid of the blood.

Immunohistochemistry of xanthine oxidoreductase

XOR was purified from human milk according to Abadeh *et al* [20]. Balb/c mice were immunised with purified human milk XOR or commercially available bovine milk XOR (Biozyme Laboratories, Blaenavon, UK). Mouse monoclonal antibodies of IgG class to human and of IgM class to bovine XOR were produced [30,31]. Fresh pieces of human and rat ventricle were immediately fixed in formalin, embedded in paraffin, and cut in 5µm sections. these were mounted to slides with 0.13 % agar. Paraffin was removed from the sections with xylene and ethanol. Endogenous peroxidase activity was inhibited by incubation with 0.5% H_20_2 in methanol for 30 min. Slides were rinsed with phosphate-buffered saline (PBS), pH 7.4 3 x 5min. XOR was detected via the avidin-biotin-peroxidase complex method using ABC peroxidase mouse IgM and IgG staining kits (Pierce Europe, Oudbeijerland, The Netherlands) [32]. To reduce nonspecific staining, slides were incubated in normal goat serum for 20 min. After excess serum was wiped off, incubation for 60 min. with the primary antibody mouse anti-(bovine XOR) or mouse anti-(human XOR) started immediately. All incubations were carried out at room temperature. Slides were washed 3 x 5 min. in PBS, and incubated for 30 min with the diluted secondary biotinylated anti-(mouse IgM/IgG) antibody. After the slides were washed, they were incubated with the avidin-biotin-peroxidase complex reagent for 30 min. To develop a brown colour where XOR is localised, the sections were incubated with 0.01 % H_20_2 and 0.05 % 3,3 diaminobenzidine tetrahydrochloride (Sigma, Poole, UK) in Tris-HCl, pH 7.6 for 7 min. under protection from light. After the sections were washed for 5 x 2 min with purified water (MilliQ / Millipore), they were counterstained with Mayer's haematoxylin for 0.5 min, and gently washed under tap water for 5 min. After dehydratation in 70 % , 96 % and 100 % ethanol, the sections were rapidly mounted

with entellan (Merck, Darmstadt, Germany). Finally, the slides were observed under a light microscope (Olympus BH-2) and photographs taken. Negative controls were done with PBS containing 1% bovine serum albumin (BSA, Sigma) and normal mouse serum instead of the primary antibody. As IgM and IgG negative control, antibodies of the IgG and IgG type to glucose oxidase from *Aspergillus niger* (Dako, Glostrup, Denmark) was used. Rat liver was used as positive control.

Staining of Smooth-muscle cells with anti-(α-actin) in human and rat tissue

To distinguish the smooth-muscle cell layer and the endothelial cells, these cell types were separately stained in both human and rat heart. An antibody to α-actin specific for smooth muscle cells was used. Paraffin was removed from the slides and the sections were hydrated. All incubation steps were carried out at room temperature. Endogenous peroxidase activity was inhibited by incubation with 0.5% H_20_2 in methanol for 20 min. Slides were rinsed with PBS before nonspecific staining was blocked with PBS/5%BSA for 15 min. Excess PBS/BSA was removed. The sections were exposed to the monoclonal antibody, peroxidase-conjugated rabbit immunoglobulins to mouse immunoglobulins (Dako) were subsequently used, with diaminobenzidine as substrate. The sections were counterstained with Mayer's haematoxylin. Positive control staining was done in rat colon. In addition, in all tissues, PBS controls were performed.

Staining of human endothelial cells with Ulex Europaeus agglutinin type 1

For this staining, the indirect peroxidase method, with diaminobenzidine as substrate, was used. The steps involved were carried out at room temperature. Endogenous peroxidase was inhibited by incubation in methanol/ 3% H_20_2 . The sections were incubated with the primary antibody Ulex Europaeus agglutinin type-1 (UEA-1, Dako) for 30 min. The second 30 min. incubation was with peroxidase conjugated rabbit anti-UEA-1 (Damo). The sections were counterstained with Mayer's haematoxylin. PBS and normal serum negative controls were carried out and no colour was observed. Positive control, human adventitia of small intestine, showed clearly brown staining of endothelial cells.

RESULTS

Immunohistochemistry of XOR in human and rat tissue

The monoclonal IgG antibody raised in mice to XOR from human milk and its IgM counterpart to bovine milk XOR gave similar results in both human and rat heart. After incubation with monoclonal mouse IgM anti-(bovine XOR), we clearly found immunoreactivity in myocytes and smooth-muscle cells of human myocardium (Fig. 1). Endothelial cells of small capillaries did not stain. Endothelial cells of larger capillaries exhibited staining varying between negative and clearly positive. Larger arteries showed clearly immunoreactivity in the endothelial cells, a non stained elastica interna layer, and a positive brown smooth-muscle layer (Fig. 2). We observed the same immunoreactivity staining pattern in rat heart; even the staining was similar in both species (Fig. 3).

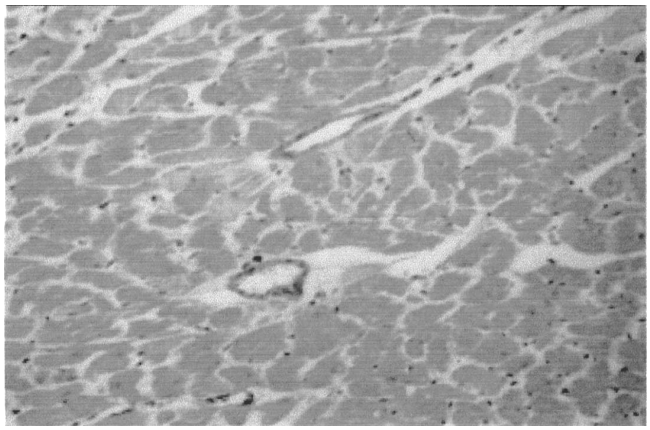

Figure 1: Human myocardial tissue incubated with monoclonal mouse IgM anti-(bovine xanthine oxidase). Myocytes and smooth-muscle cells of the blood vessels are clearly stained. The endothelial cells of the smaller vessels do not show any staining.

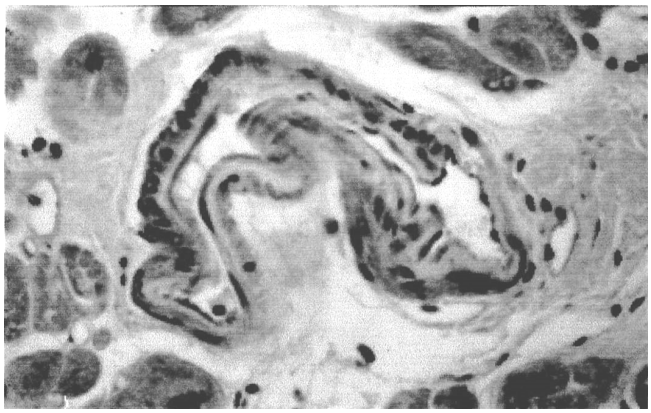

Figure 2: A larger artery in human heart, surrounded by myocytes, incubated with mouse IgG anti-(human XOR). This shows a clear immunostaining of endothelial cells, nonstained elastica interna, and immunoreactivity in the smooth-muscle layer. Surrounding myocytes also developed a strong colour.

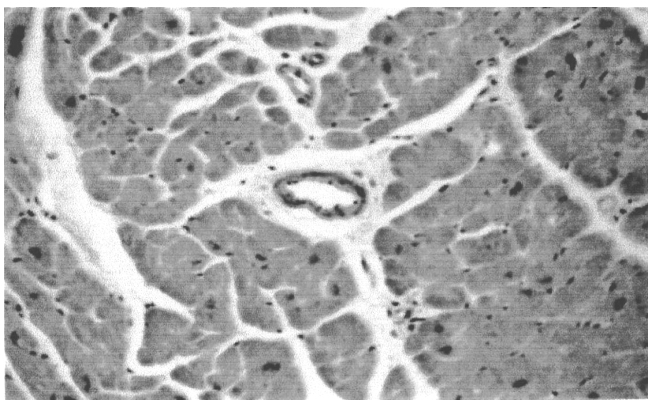

Figure 3: Rat myocardial tissue incubated with monoclonal mouse IgG anti-(human XOR). The picture shows the same pattern and intensity of staining in myocytes and smooth muscle cells as in human heart. No reaction with endothelial cells of small vessels.

Because antibodies of the IgM type sometimes cause non specific staining, a negative control was carried out using IgM antibody to glucose oxidase, from *Aspergillus niger*, and no staining was observed in both human and rat heart. Replacement of the primary antibody by normal mouse serum or PBS gave clearly negative results in both species. In the same procedure,. similar results were obtained with IgG controls.

We used rat liver as a positive control. The monoclonal IgM anti-(bovine XOR) antibody showed immunoreactivity in hepatocyte cytosol, and a more intense colour in smooth-muscle cells of the microvasculature. Controls, IgM anti-glucose oxidase, PBS and normal serum, were negative.

Staining of human endothelial cells with Ulex Europaeus agglutinin type 1

In human heart we outlined the endothelial cells of the smallest blood vessels with UEA-1 (Fig. 4). Note that the antibodies against human and bovine milk XOR did not stain these capillaries.

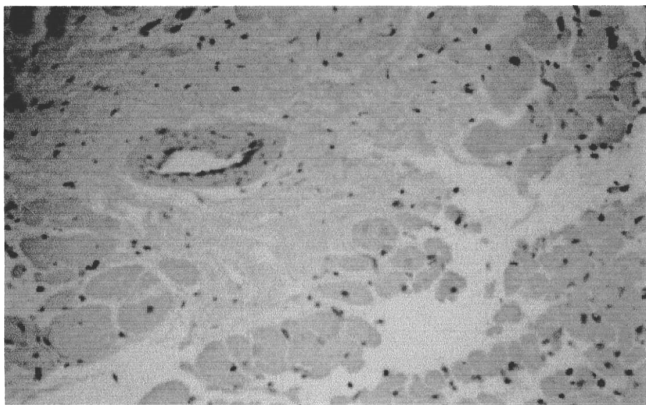

Figure 4: Staining with Ulex Europaeus agglutinin type 1 (UEA-1) of human myocardial tissue. UEA-1 clearly shows endothelial cells. Note that small capillaries stain well in contrast to the immunoreactivity with XOR antibodies.

DISCUSSION

Immunohistochemistry

Jarasch *et al*. [15] were first to report, briefly, the immunohistochemical localisation of xanthine oxidase in human heart with antibodies purified from human serum. they found the enzyme mainly in capillary endothelial cells. Moriwaka *et al*. [17] also reported that endothelial cells of human coronary artery reacted immunopositive to the polyclonal anti-XOR antibodies (the enzyme used for immunisation was purified from human liver). Both studies failed to mention the sources and status of the human tissue used.

Several investigators purified XOR from human milk and raised antibodies to the enzyme [14, 33]. Hellsten-Westing described the presence of XOR in smooth-muscle and endothelial cells [14]. Sarnesto *et al*. [25] reported briefly that their antibody failed to react with human heart in Western blots. With monoclonal and polyclonal antibodies to the enzyme, purified from human milk, cardiomyocytes, smooth-muscle cells and a varying number of endothelial cells were stained clearly for XOR for both species (human and rat).

Literature data and our observations show considerable variation in immunohistochemical reactivity and localisation of XOR. These discrepancies could be ascribed to differences in (**i**) enzyme preparation used to raise antibodies; (ii) the antibodies isolated (monoclonal versus polyclonal, epitope variation etc); (iii) detection system (e.g. secondary antibody); (iv)human heart tissue studied (disease state, tissue collection and storage).

Failure to detect xanthine oxidase activity

A variety of sensitive techniques failed to detect XOR activity in human heart [10-12, 18). If we ignore the older case studies, only Wajner and Harness [13] detected and claimed high activity in human heart. We fail to have an explanation for these anomalous results. The low activity of the enzyme in human heart should be reconsidered in the light of an endogenous high molecular weight inhibitor, recently described in rabbit heart [34]. Furthermore, the existence of isoforms, such as inactive desulpho-XOR and demolybdo-XOR forms, deserves more attention.

Isoforms

The presence of inactive demolybdo and desulpho XOR isoforms, in addition to the oxidase and dehydrogenase forms, has been known for a long time [27]. About 70% of total XOR in the milk and about 40 % of total XOR in the liver is in the inactive desulpho form [29,31,36]. Furthermore, purification procedures may cause the production of 20 to 40 % desulpho-enzyme from bovine milk [27]. Sulphur transferases, such as rhodanese, can activate the desulpho form in many tissues, including heart [38-40].

As described previously [23], human milk XOR may occur in the 'inactive' desulpho form. We have evidence that likewise human heart has a similar property [41]. We want to emphasise that the inactivity of this form refers to the conversion of (hypo) xanthine to urate. desulpho-XOD can react with endogenous NADH and produce superoxide radicals.

Implications

Various investigations, including the present study, have demonstrated that xanthine oxidase may occur in human heart. Its expression in endothelial and other cells seems to vary, and may be related to disease states causing activation of endothelial cells [42]. We found clear evidence of its presence in monocytes and smooth-muscle cells. Since human heart may contain the desulpho form, the question whether xanthine oxidase can damage human heart tissue by free radical generation remains open.

Limitations of the study

Although we tried to use human heart as fresh as possible, it had to be arrested and explanted. Based on rat heart studies [7], we do not believe that cardioplegia affected the XOR content of human heart samples. However, we cannot exclude that heparin used in our

patients has led to XOR depletion. Note that human XOR has an affinity for heparin and can bind via its lysine and/or arginine residues to endothelial cells [43], It has been shown that heparin removes endothelium XOR [44].

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