Punjab Univ. J. Zool., Vol. 13, pp. 89-98, 1998

DISTRIBUTION OF XANTHINE OXIDASE DURING CALCIUM PARADOX CONDITIONS OF RAT HEART

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Abstract: Reintroduction of calcium into calcium depleted rat heart, so called Ca-paradox (CP), results in extensive biochemical, physiological and histological changes. Free radical producing enzyme xanthine oxidase (XO) was localized (*in situ*) in PLP fixed thick frozen sections of normal and Ca-paradox rat hearts by enzyme histochemistry using nitroblue tetrazolium (NBT) staining and also by immunocytochemistry using murine monoclonal antibodies against XO. An immunoperoxidase reaction product was accumulated in interstitial cells, endothelial cells of small capillaries and blood vessels in normal rat heart whereas it was markedly increased in Ca-paradox tissues, but XO activity was reduced by nitroblue tetrazolium staining which suggested the loss of enzyme activity. The results indicate that the XO activity during Ca-paradox conditions is increased and is restricted to interstitial and endothelial cells and its activity in sarcolemma membranes is due to rupturing and shedding of the cytoplasmic materials of interstitial and endothelial cells. The activity of XO along with CP may enhance the myocardial abnormalities.

Key words: Calcium paradox, XO, immunocytochemistry, endothelial cells, myocardiam.

INTRODUCTION

The term "Calcium Paradox" (Ca-paradox) illustrates the reintroduction of medium containing Ca^{+2} after a brief period of perfusion with Ca^{+2} free medium. This process leads to the deterioration of structure and function, extensive biochemical, physiological and histological changes (Yate and Dhalla, 1975; Frank, 1983; Ganote *et al.*, 1983; Hulsmann, 1983) and ultimately loss of mechanical functions occur.

Calcium ions play an important role in physiological and pathological processes but the exact mechanism, which proceed the development of myocardial abnormalities are far from clearly understood. However, it is only one typical form of myocardial injury caused by excessive Ca^{+2} engulfment (Nayler *et al.*, 1983).

During pathological conditions, high energy phosphate level (ATP) is rapidly channeled to concomitant increase of ADP, AMP and purine bases, which serve as a fuel for xanthine oxidase (XO). This enzyme is mainly coupled to the degradation of xanthine and hypoxanthine to urates by producing a bulk of highly reactive cytotoxic

0079-8045/98/0001-0089 $ 03.00/0

superoxide radicals (Bray, 1975; Weiss, 1986) which are involved in protruding the disturbed normal physiological functions. However, the biological role of these radicals has not yet been completely known. Xanthine oxidase is normally present as dehydrogenase form (called D-type) and utilizes NAD^+ as electron acceptor (Park and Ganger, 1986) but under certain conditions, it rapidly converts into oxidase form (called O-type) and transfer electron to oxygen molecule (McCord, 1987; Schoutsen *et al.*, 1983). A number of studies (Krenitsky *et al.*, 1975) was attempted to elucidate XO activity in different organs of animals but no information is available as to which organelles of rat heart show active sites of XO during Ca-paradox conditions. It is important to examine the role of XO in myocardial abnormalities associated with Ca-paradox.

The present study was conducted to localize the XO activity in Ca-paradox rat heart using immunocytochemical methods at light and electron microscopic level and compared with enzyme histochemistry. The ultrastructural changes occurring during pathological stages have also been discussed.

MATERIALS AND METHODS

Monoclonal antibody

Murine MAb (N_2-26) against XO was characterized by enzyme linked immunosorbant assay (ELISA) and immunoblot. After checking the specificity and titer of MAb, it was used for immunolocalization studies in normal and Ca-paradox rat hearts.

Materials required for propagation of hybridoma (N2-26) secreting monoclonal antibodies (MAbs) against XO and for immunochemical tests were described in Samra *et al.* (1991). All other chemicals for routine analysis were obtained from Sigma Chemical Company.

Perfusion techniques

Normal rat heart

After being heparinized (5000 USP) and anesthetized with pentobarbitol (30 mg/kg), Sprauge-Dawley male rat (weighing 200-250 g) hearts were excised and perfused retrogradely in a non-circulating langendorff apparatus (Samra *et al.*, 1991) with Krebs-Henseleit buffer, saturated with 95% O_2 and 5% CO_2 for 15 minutes at 37°C. Perfused hearts were fixed immediately with cold periodate-lysine-paraformaldehyde (PLP) fixative in 0.4M phosphate buffer (pH 7.4) and stored at 4°C for further studies.

Calcium paradox rat heart

Again after being heparinized and anesthetized, rat hearts were removed and perfused under normal conditions as described above, followed by 10 minutes of calcium free perfusion and then 10 minutes reperfusion with buffer containing 2.5mM calcium. Treated hearts were fixed with cold PLP fixative and stored at 4°C for further studies.

Tissue embedding and homogenate preparation

Fixed and sucrose (15%) treated tissues from normal and calcium paradox rat hearts were embedded in Optimum Cutting Temperature (OCT) compound, snap frozen in dry ice ethanol bath and then stored frozen at -70°C for light and electron microscopic studies. Hearts subjected to Ca-paradox were also processed for homogenate preparation (Samra *et al.*, 1991). This homogenate was used to detect the XO activity and immunoblot analysis.

Immunoblot analysis

Purified XO and Ca-paradox rat heart homogenate were separated electrophoretically in slab sodium dodecyl sulphate-polyacrylamide gel (10%). After electrophoresis, protein samples were transferred electrophoretically on to nitrocellulose membrane (Towbin and Gordon, 1979) and proceeded for immunochemical reaction.

Immunocytochemical studies

For light and electron microscopic studies, thick cryostat sections of frozen tissues were processed for immunoperoxidase labelling (Samra *et al.*, 1991; Ashraf and Samra, 1993). For light microscopy, cryosections (6-8 μm) were cut, placed on albumin coated slides and exposed to MAbs (mouse anti-XO) to arrest its epitopes. The bound MAbs were again allowed to react with peroxidase labelled goat anti-mouse IgG. After washing, immunochemical reaction was developed by incubating the sections with 0.2% diaminobenzidine and 0.05% H_2O_2 in 0.05M Tris-HCl (pH 7.4). Rinsed tissues were dehydrated in ascending series of ethanol up to absolute ethanol and mounted in permount.

For electron microscopic studies, PLP fixed rat heart tissues were dehydrated in ascending series of ethanol. Dehydrated tissues were embedded in plastic resin (Epon) and subsequently polymerized at 60°C for 48 hour. Ultra-thin sections were cut, placed on copper grids and processed for immunoperoxidase labelling (described above) and observed with Hitachi electron microscope at accelerations voltage of 75KV. Control sections were treated with HT-medium, or secondary antibody alone.

Enzyme histochemistry

Enzyme activity (*in situ*) in normal and Ca-paradox rat hearts was also checked as described in Samra *et al.* (1991). Briefly, PLP fixed 6-8 μm sections of normal and Ca-

paradox rat heart tissues were incubated in a reaction medium (0.05M Tris-HCl, pH 7.4; 1mM Hypoxanthine; 1 mg/ml Nitroblue tetrazolium) for 20 minutes. For control sections, 1mM allopurinol (an inhibitor of XO) was also added in the reaction medium.

RESULTS

Characterization of antibodies

ELISA and immunoblot analysis showed that the murine MAbs against XO recognized the epitopes present on native XO. The specificity and monoclonality against pure XO as well as in rat heart homogenate were further examined under denaturing conditions (Fig.1). When purified XO and Ca-paradox rat heart homogenate (105,000 xg) supernatants were processed for immunoblot, a specific immune reaction with 155 KDa protein band was detected on nitrocellulose membrane. However, an additional protein bands of approximately 91 KDa in pure XO and two other protein bands of approximately 60 KDa and 27 KDa in addition to 91 KDa in rat heart homogenate were also visualized with weak staining. Immunochemical controls incubated with Hypoxanthine-Thymidine Dulbeco Modified Eagles Medium (HT-DMEM) or secondary antibody alone were devoid of reactivity.

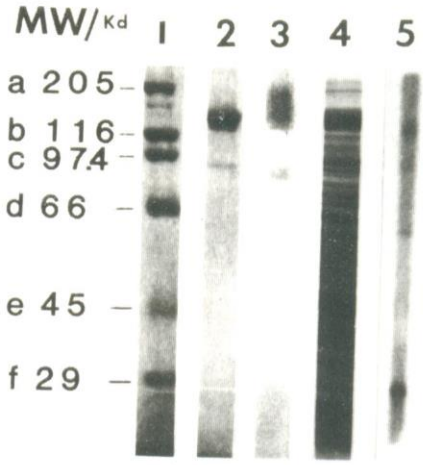

Fig. 1: **Immunoblot analysis of XO. Lane 1, standard molecular weight: (a) myosin, 205 KDa; (b) ß-galactosidase, 116 KDa; (c) phosphorylase-b, 97.4 KDa; (d) bovine serum albumin, 66 KDa; (e) ovalbumin, 45 KDa; (f) carbonic anhydrase, 29 KDa. Lane 2, pure XO. Lane 4, Ca-paradox rat heart homogenate; Lane 3 & 5, immunodetection of pure XO and also in rat heart homogenates, respectively.**

Light microscopic observation

Immunoperoxidase staining

Thick cryosections of normal and Ca-paradox rat hearts tissues subjected for immunoperoxidase labelling showed that the dark brown color reaction product was precipitated on antigenic sites. Tissue sections from normal perfused heart (Fig.3) showed weak immunostaining on interstitial cells between muscle fibres. Whereas tissue sections of rat heart underwent for Ca-paradox were signalling a copious amount of immunoreaction product distributed intensely on interstitial cells (Fig.2a), small capillaries and blood vessels (Fig.2b) along with weak immunoreaction product on heart muscles.

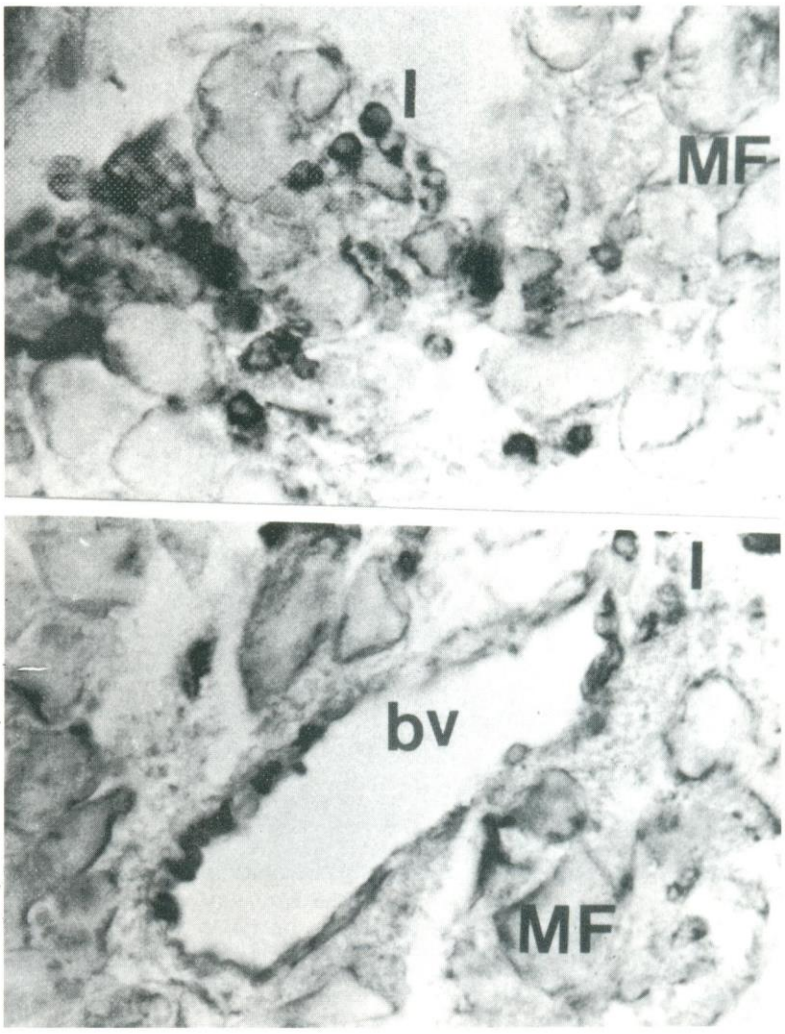

Fig. 2: An immunoperoxidase staining on frozen sections of Ca-paradox rat heart incubated with MAb against XO. The reaction product is seen on (*2a*), Interstitial cells (I), muscle fibres (MF), (*2b*), blood vessels (bv), (X 350).

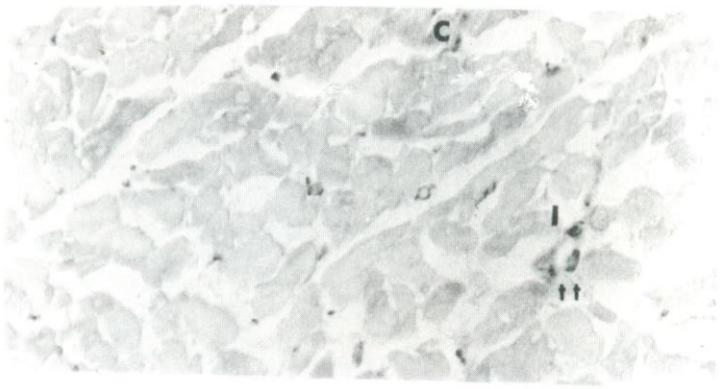

Fig. 3: **An immunoperoxidase staining on frozen sections of normal rat heart incubated with MAb against XO. A reaction product is seen on capillaries (c), interstitial cells (I), (X 250).**

Nitroblue tetrazolium staining

Cryosections of Ca-paradox rat heart were also analyzed for XO activity (*in situ*) by nitroblue tetrazolium (NBT) staining. It was observed that insoluble blue formazan reaction product was deposited on small capillaries and interstitial cells along with weak staining on muscle fibre (Fig.4).

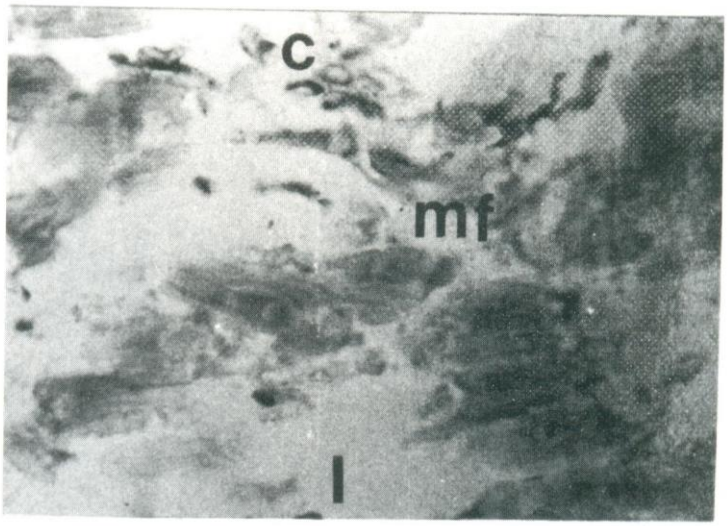

Fig. 4: **Nitroblue tetrazolium staining on frozen sections of Ca-paradox rat heart. A blue formazan staining on capillaries (c), interstitial cells (I), muscle fibres (mf), (X 350).**

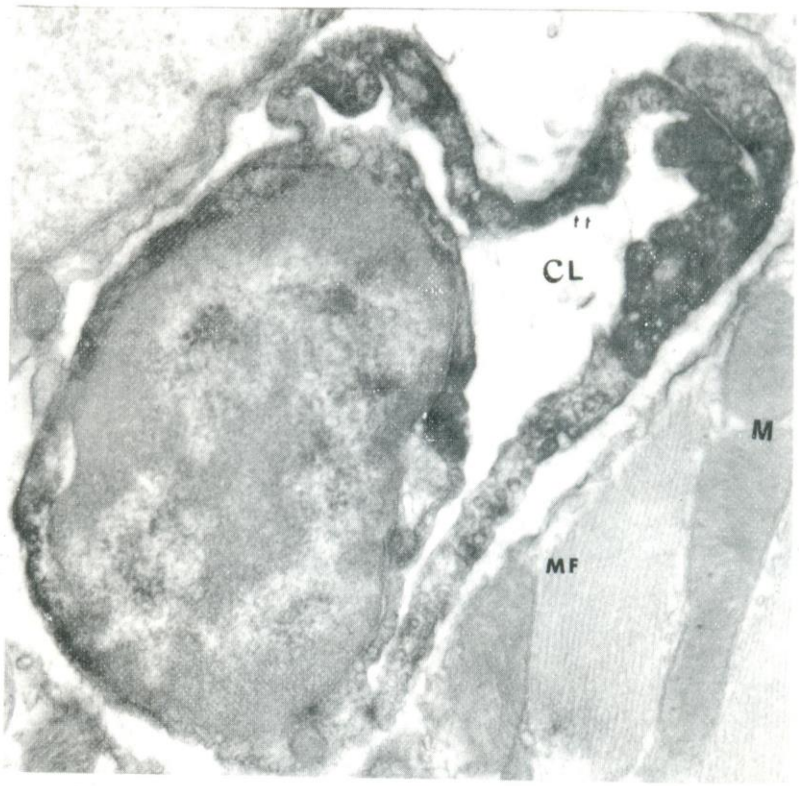

Fig. 5: **Immunoelectron microscopy of XO in Ca-paradox rat heart. The reaction product is located on (*5a*), interstitial cells (I), ruptured surrounding capillary lumen (CL), partial separated sarcolemma (S), swollen mitochondria (M) are also seen (X 8000).**

Electron microscopic observation

Observation of ultra-thin sections of epon-embedded normal and Ca-paradox rat hearts showed that the diamino benzidine (DAB) reaction product was deposited in the cytoplasm of interstitial cells, cytoplasmic rim surrouding the capillary endothelium of normal heart (Fig.6) as well as ruptured cytoplasmic rim in Ca-paradox rat heart but the staining was markedly increased in Ca-paradox conditions (Fig.5). A weak immunostaining was also observed only on the adjacent heart muscle fibres in Ca-paradox tissues. Mitochondria, nuclei of endothelial cells and interstitial cells were also not stained.

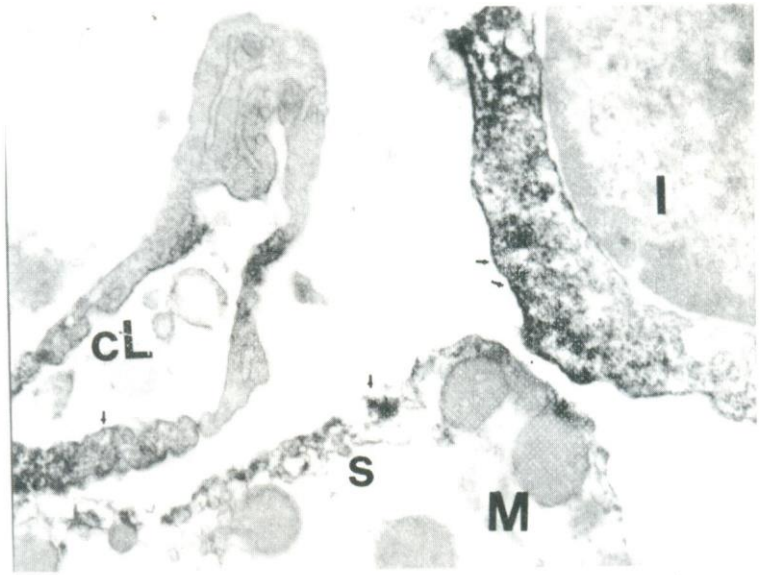

Fig. 6: **Immunoelectron microscopy of XO in normal rat heart. The reaction product (arrow head) is located on surrounding capillary lumen (CL), but muscles fibres (MF), and mitochondria (M) are not stained (X 8000).**

Morphological studies

The morphological studies of calcium reperfused rat heart tissues at light and electron microscopic level showed the severe damage of muscle fibres, partial separation of outer lamina of glycocalyx of sarcolemma, contraction of myocardial cells, swollen mitochondria and ruptured intercalated disc membrane.

Immunocytochemical control

All control sections proceeded for light and electron microscopic studies did not show staining.

DISCUSSION

In the present study, the activity of murine MAbs against XO was exploited to explicate the role of XO in normal and Ca-paradix rat hearts. The use of MAbs is superior to polyclonal antibodies due to its supreme precise reactions. The

monoclonality was corroborated with ELISA, and immunoblot analysis under native and denatured conditions respectively which encouraged for immunocytochemical studies to determine the active sites of XO in Ca-paradox rat heart.

Immunoblot analysis revealed more specific bindings of MAbs to 155 KDa protein band of pure XO as well as in Ca-paradox rat heart homogenates. Whereas the immunochemical reaction with 91 KDa in pure XO and 60 KDa, 29 KDa protein bands in addition to 91 KDa bands in rat heart homogenate suggested the breakdown polypeptide of enzyme during storage or homogenate preparation of rat heart respectively.

Immunocytochemical and enzyme histochemical techniques were used to explore the XO at light and electron microscopic level using immunoperoxidase and NBT staining, which are mainly depend upon the presence of epitopes and preservation of functional property of the enzyme respectively. An immunoperoxidase analysis at light and electron microscopic level documented the presence of XO in normal rat hearts but its activity markedly increased after reperfusion with calcium containing buffer. A weak immunostaining on adjacent muscle fibres of tissues from Ca-paradox suggested that this may be due to rupturing and shedding of the cytoplasmic materials of these cells. This finding was not seen in our previous studies (Samra *et al.*, 1991; Ashraf and Samra, 1993) which illustrated lack of immunostaining on muscle fibres during pathological conditions such as ischemia or ischemia/reperfusion.

Xanthine oxidase activity was also monitored spectrophotometrically in rat heart homogenates by detecting the conversion of hypoxanthine to uric acid at 292 nm (data not shown). In contrast to immunocytochemistry, a weak histochemical (*in situ*) staining by NBT convinced the loss of XO activity during Ca-paradox conditions. Whereas normal tissues showed the moderate enzyme activity. Allopurinol is considered as an inhibitor of XO activity and sections of normal and Ca-paradox heart tissues with this inhibitor were lack of enzyme activity. The behaviour of immunochemical activity over enzyme activity will be more reliable to make out the active sites of this enzyme.

The morphological studies by electron microscopy revealed that the Ca-paradox annulled the normal functions of heart and ultimately lost its activity. Although enzyme histochemical and immunocytochemical approaches were applied to explore the XO activity in many tissues (Park and Granger, 1986) but no data was available about the precise localization of this enzyme in Ca-paradox rat heart.

The observations in the present study suggested that the Ca-paradox induces disturbance in the xanthine dehydrogenase/ oxidase ratio and may enhance the production of oxygen derived free radicals. The presumed conversion of dehydrogenase to oxidase form in Ca-paradox presents a very important step in the series of events leading to cellular necrosis.

The results reveal the augmented activity of XO is restricted on interstitial cells and endothelial cells of small capillaries and blood vessels. The staining on adjacent muscle fibres may be due to rupturing and shedding of cytoplasmic materials of these cells. It is

concluded that oxygen free radicals produced by action of XO along with Ca-paradox condition may promote severe damage of the activity of heart muscles.

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