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Evidence of Hydroxyl Free Radical Generation by Calcium Overload in Rat Myocardium

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Abstract

Although calcium (Ca^{2+}) is important in cardiac dysfunction and has also been reported as a source of oxidative toxicity, the connection between Ca^{2+} overload and oxygen free radicals in the myocardium is not clear. We have investigated whether Ca^{2+} overload generates hydroxyl free radicals in rat ventricle.

HPLC with electrochemical detection was used to measure the levels of 2,3- and 2,5-dihydroxybenzoic acid (DHBA) formed when the hydroxyl free radical reacts with salicylate. Ringer's solution containing salicylic acid (0·5 nmol μL^{-1} min^{-1}) was infused through a microdialysis probe in the region of the left anterior descending coronary artery of the rat ventricle. A positive linear correlation was obtained between Ca^{2+} and hydroxyl free radical formation trapped as 2,3-DHBA ($r^2 = 0{\cdot}976$) and 2,5-DHBA ($r^2 = 0{\cdot}982$) in the myocardial dialysate. The administration of ouabain (1 mg kg^{-1}, i.v.), a Ca^{2+} elevator, into the femoral vein significantly increased the level of 2,3- and 2,5-DHBA.

These results indicate that Ca^{2+} overload generates hydroxyl free radicals in rat heart.

The occurrence of intracellular calcium plays a key role in cardiac dysfunction. Intracellular calcium (Ca^{2+}) has been reported as a source of oxidative toxicity (Bellamo et al 1989). Ouabain, which inhibits the sodium–potassium pump, resulting in dissipation of the sodium gradient and leading to increased levels of cytosolic free Ca^{2+} and total Ca^{2+} (Murphy et al 1985), has been used to induce Ca^{2+} overload (Patmore et al 1989; Cousin et al 1995). Elevation of intracellular Ca^{2+} levels causes the conversion of xanthine dehydrogenase to xanthine oxidase (Vannuci 1990), which produces superoxide. However, the connection between intracellular Ca^{2+} overload and oxygen free radicals in the myocardium is not clear.

The overproduction of reactive oxygen, such as O_2^-, H_2O_2 and the hydroxyl free radical can cause cellular injury. The hydroxyl free radical is extremely reactive and readily reacts with a number of compounds, including lipids, proteins and nucleic acids (Das et al 1989; Pou et al 1989). Hydroxyl free radicals react with salicylate and generate 2,3- and 2,5-dihydroxybenzoic acid (Grootveld & Halliwell 1986; Halliwell et al 1991), which can be measured electrochemically in picomole quantities by high-performance liquid chromatography (HPLC) (Radzik et al 1983; Floyd et al 1984; Obata & Chiueh 1992). In the current study we have used salicylate hydroxylation as a probe (Floyd et al 1984; Powell & Hall 1990) to monitor the course of formation of hydroxyl free radicals in rat myocardium by use of microdialysis (Obata et al 1994; Obata & Yamanaka 1996). The study examined whether calcium overload generates hydroxyl free radicals in rat ventricle.

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Materials and Methods

Experimental protocol

Adult male Wistar rats, 300–400 g, were kept in an environmentally controlled room (20–23°C, 50–60% humidity, illuminated from 0700 to 1900 h). The rats were anaesthetized with chloral hydrate (400 mg kg^{-1}, i.p.; Sigma, USA) and the level of anaesthesia was maintained by continuous intravenous infusion of chloral hydrate (20 mg kg^{-1} h^{-1}). Artificial ventilation was maintained by a constant-volume respiration system using oxygen–mixed air. The heart rate, arterial blood pressure and electrocardiogram were monitored and recorded continuously. Arterial blood pH, pO_2, pCO_2 and core body temperature were maintained within normal ranges. The study was approved by the Ethical Committee for Animal Experiments, Oita Medical University.

We have designed a system for holding the microdialysis probe which enables loose fixation of the probe and its synchronized movement with each motion of the organ under investigation (Obata et al 1994). The dialysis probe was implanted in the region of the left anterior descending coronary artery. Heparin sodium (200 units kg^{-1}) was administered intravenously before probe implantation and 100 units kg^{-1} was then given every hour to prevent blood coagulation. When a perfusion flow of 1 μL min^{-1} was used, the relative recovery rate of a 1-μM standard solution of 2,3-dihydroxybenzoic acid (DHBA) and 2,5-DHBA was approximately 10 and 11%, respectively. Because the noradrenaline level in the dialysate reached a steady state 150 min after probe implantation, we started the measurements of 2,3- and 2,5-DHBA at this time.

Ouabain, sodium chloride, and sodium salicylate and its hydroxylated metabolites were purchased from Sigma, USA.

The probe was washed with Ringer's solution containing 147 mM NaCl, 2·3 mM $CaCl_2$ and 4 mM KCl (pH 7·4) for at least 30 min before probe implantation in the myocardium; infusion was then continued (1 μL min^{-1}) for at least 150 min before switching to experimental drug solution by means of a liquid switch. Hydroxyl free radicals were subsequently trapped by perfusing the myocardium with 0·5 mM sodium salicylate in Ringer's solution (0·5 nmol μL^{-1} min^{-1}) for 110 min. Samples (1 μL min^{-1}) were collected every 10 min into small collecting tubes containing $HClO_4$ (0·1 M; 10 μL).

Analytical procedure

The dialysate samples were immediately analysed for 2,3- and 2,5-DHBA by HPLC fitted with a 4·6×150 mm×5 μm Eicompak MA-5ODS reversed phase analytical column and equipped with an electrochemical detector (Eicom, Japan). The glassy carbon working electrode was set at a potential of 0·75 V. The mobile phase contained heptanesulphonic acid sodium salt (1·5 g L^{-1}; Sigma), Na_2EDTA (0·1 g L^{-1}), triethylamine (3 mL L^{-1}; Wako Pure Chemical Industries, Japan) and acetonitrile (125 mL L^{-1}; Wako) dissolved in H_2O. The pH of the solution was adjusted to 2·8 with 3 mL phosphoric acid (Wako) and filtered through a Millipore filter before addition of the acetonitrile. The flow rate was between 0·7 and 0·9 mL min^{-1}. The results were expressed as the mean ± s.e. Differences between the time courses of the levels of 2,3- and 2,5-DHBA were studied statistically by means of the Mann–Whitney *U*-test.

Results

Cumulative dose–response for the formation of 2,3-DHBA by Ca^{2+}

In a cumulative dose–response experiment four different concentrations of Ca^{2+} ($2·3\times10^{-3}$, 2×10^{-2}, 4×10^{-2} and 6×10^{-2} M) were infused directly through a microdialysis probe into the rat myocardium for 10 min each. Ca^{2+} clearly induced a dose-dependent increase in the formation of hydroxyl free radicals; a positive linear correlation was obtained between Ca^{2+} and hydroxyl free radical formation trapped as 2,3-DHBA ($r^2=0·976$) and 2,5-DHBA ($r^2=0·982$) in the dialysate (Fig. 1). The retention times of authentic standards of 2,3- and 2,5-DHBA were identical with those of the compounds in the dialysate.

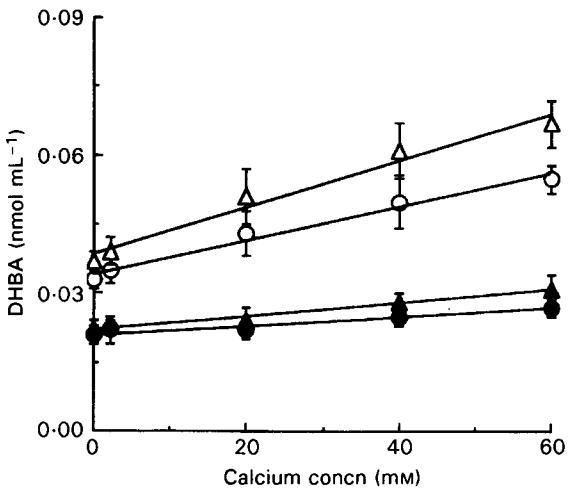

FIG. 1. Cumulative dose-response relationship between Ca^{2+} formation and the efflux of 2,3- and 2,5-DHBA in the heart. After a 150-min wash with Ringer's solution, pH 7·4, Ca^{2+} and sodium salicylate (0·5 nmol μL^{-1} min^{-1}) were infused directly through the microdialysis probe into the rat myocardium. The dialysate samples for the determination of 2,3- (△) and 2,5-DHBA (○) were collected every 10 min and assayed immediately by HPLC with electrochemical detection. The linear correlation coefficients for 2,3- and 2,5-DHBA were $r^2=0·976$ and $r^2=0·982$, respectively. Corresponding experiments were performed with the probe as reagent blank of 2,3- (▲) and 2,5-DHBA (●). Values are means ± s.e. from six rats.

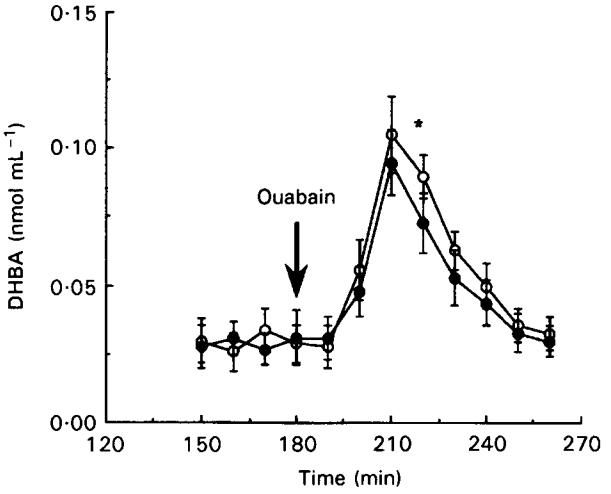

FIG. 2. Effect of ouabain (1 mg kg^{-1}, i.v.) on the formation of hydroxyl free radicals in the myocardium of rats. Cardiac microdialysis heart perfusion was used to monitor the time-course of in-vivo trapping of highly reactive hydroxyl free radicals in the extracellular fluid of the myocardium. After a 150-min wash with Ringer's solution, pH 7·4, sodium salicylate (0·5 nmol μL^{-1} min^{-1}) was infused directly through the microdialysis probe into the rat myocardium for 110 min. The administration of ouabain (1 mg kg^{-1} i.v.) induced an increase in the formation of 2,3-DHBA (●) and 2,5-DHBA (○). Differences in time course between 2,3- and 2,5-DHBA levels were statistically studied by means of the Mann–Whitney U-test. Values are expressed as mean ± s.e. from six rats. $*P<0·05$, significantly different compared with steady-state levels (150–160 min). Abscissa: infusion of salicylic acid was started after 150-min wash.

Induction of hydroxyl free radical formation by ouabain

When ouabain (1 mg kg^{-1}) was administered via the femoral vein and sodium salicylate was infused for 110 min to trap hydroxyl free radicals, a marked elevation in the levels of 2,3- and 2,5-DHBA was observed in the heart dialysates (Fig. 2). The level of DHBAs increased three-fold in the 30 min after ouabain administration and then decreased gradually. The retention times of 2,3- and 2,5-DHBA were again determined by use of authentic samples. Elevation of the levels of 2,3- and 2,5-DHBA was not observed after administration of a low dose of ouabain (0·5 mg kg^{-1}). Changes typical of ouabain were observed in the electrocardiogram.

Discussion

Although the participation of sodium–calcium exchange in the mechanism of intracellular Ca^{2+} elevation has been suggested (Murphy et al 1985), detailed information about the relationship between the status of sodium–calcium exchange and oxygen free radical formation has not been available. The occurrence of intracellular Ca^{2+} overload plays an important role in cardiac dysfunction. Calcium ions might be a very

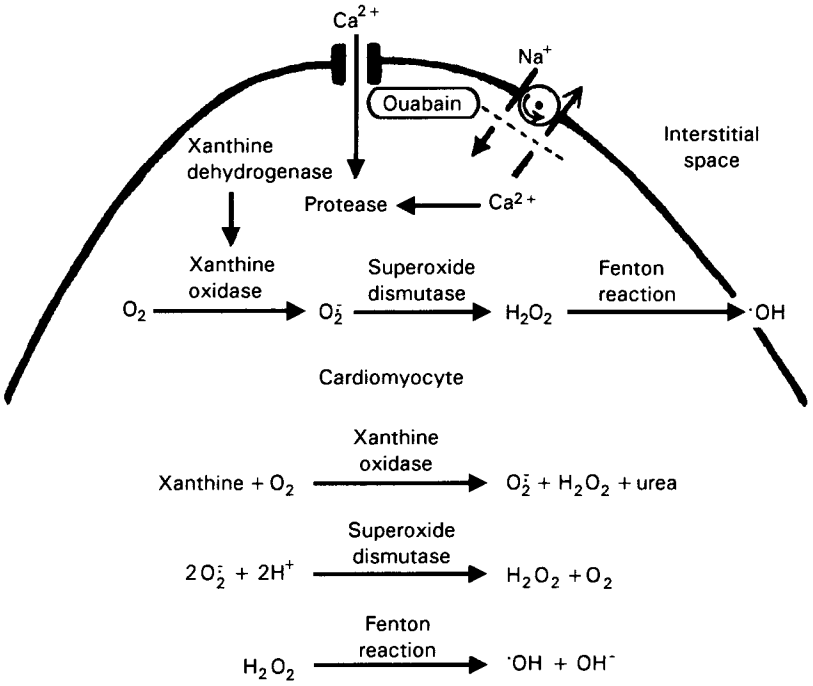

FIG. 3. Reaction pathway illustrating the formation of hydroxyl free radicals by ouabain in rat heart. O_2^-, superoxide anion; ·OH, hydroxyl radical.

important factor in induction of irreversible ischaemic injury (Shen & Jennings 1972). Ouabain inhibits the sodium-potassium pump resulting in dissipation of the sodium gradient which leads to an increase in cytosolic free calcium and a rise in total calcium (Murphy et al 1985). Ouabain has been used to cause a Ca^{2+} overload condition (Patmore et al 1989; Cousin et al 1995). Increased intracellular Ca^{2+} concentrations cause superoxide production (Chacon & Acosta 1991). The intravenous administration of 1 mg kg^{-1} ouabain increased the levels of 2,3- and 2,5-DHBA in the heart dialysate. Although the condition of the method is not physiological, it is expected that extracellular calcium would follow its concentration gradient and subsequent intracellular calcium overload would ensue. Therefore, calcium ions increased dose-dependently the formation of 2,3- and 2,5-DHBA; a positive linear correlation was obtained between Ca^{2+} and hydroxyl free radical formation in the myocardial dialysate (Fig. 1). Free radical formation might have contributed to in-vivo formation of free radicals by Ca^{2+}.

Intracellular Ca^{2+} overload has been reported to be one of the causes of ischaemic reperfusion injury (Hess & Manson 1984). Ouabain clearly induced an increase in hydroxyl free radical formation. However, the exact mechanisms leading to the development of this abnormality are far from clear. The enzyme xanthine oxidase is thought to be a source of superoxide anion radical (O_2^-). During the inflow of a large amount of oxygen during reperfusion, xanthine oxidase catalyses the conversion of hypoxanthine to xanthine and simultaneously generates O_2^- (Marubayashi et al 1991). O_2^- itself is poorly reactive in aqueous solution, but does participate in reactions involving iron ions and leads to the generation of more damaging hydroxyl free radical species. The hydroxyl free radical might be formed in-vivo during enzymatic reactions (Fig. 3). O_2^- has an extremely short half-life (Halliwell 1989) and rapidly undergoes dismutation yielding H_2O_2. H_2O_2 then undergoes a Fenton-type reaction in the presence of iron and yields the highly cytotoxic hydroxyl free radical (Ben-Shachar et al 1991; Gerlach et al 1994). The hydroxyl free radical can also arise from interaction between H_2O_2 and O_2^- (Haber-Weiss reaction). These results indicate that calcium overload might generate hydroxyl free radicals in rat heart (perhaps via an indirect mechanism). Free radical reactions are a part of normal metabolism in man. When produced in excess, radicals can cause tissue injury. The results of this study might be useful in elucidating the actual mechanism of free radical formation in heart disorders such as myocardial infarction.

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