Effects of Exogenous Free Radicals on Electromechanical Function and Metabolism in Isolated Rabbit and Guinea Pig Ventricle

Implications for Ischemia and Reperfusion Injury

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Abstract

Oxygen-derived free radicals have been implicated in the pathogenesis of cardiac dysfunction during ischemia, postischemic myocardial "stunning," and reperfusion injury. We investigated the effects of oxygen-derived free radicals on cardiac function in intact isolated rabbit hearts and single guinea pig ventricular myocytes. In the intact rabbit ventricle, exposure to free radical–generating systems caused increased cellular K^+ efflux, shortening of the action potential duration, changes in tension, and depletion of high energy phosphates similar to ischemia and metabolic inhibition. In patch-clamped single ventricular myocytes, free radical–generating systems activated ATP-sensitive K^+ channels, decreased the calcium current, and caused cell shortening by irreversibly inhibiting glycolytic and oxidative metabolism. The results suggest that free radicals generated during ischemia and reperfusion may contribute to electrophysiologic abnormalities and contractile dysfunction by inhibiting glycolysis and oxidative phosphorylation. Inhibition of metabolism by free radicals may be an important factor limiting functional recovery from an ischemic insult after reestablishment of effective blood flow.

Introduction

Reperfusion of cardiac tissue after prolonged ischemia is associated with persistent electromechanical and metabolic dysfunction, including cellular K^+ loss, reduced tension development, elevated rest tension, and depressed levels of high energy phosphates (1, 2). These abnormalities may be caused by direct damage to cellular structures such as the sarcolemma and sarcoplasmic reticulum, which are important in the regulation of intracellular volume and calcium concentration. Damage to the metabolic machinery is also a major factor limiting the potential for eventual recovery of electromechanical function. As recently reviewed (3), oxygen-derived free radicals have been implicated in the pathogenesis of ischemic and postischemic reperfusion injury in the heart. In vitro studies have demonstrated that free radical species accumulate to supernormal levels during experimental myocardial ischemia and especially during reperfusion (4). Products of lipid peroxidation such as malondialdehyde also accumulate, providing indirect evidence of free radical–induced cellular damage (5). Free radical–generating systems impair the function of several cardiac structures, including the sarcoplasmic reticulum (6), Na^+-K^+ pump (7), myofibrillar ATPase (8), and mitochondria in vitro (9). Free radical scavengers, including SOD and catalase (CAT),[1] in vitro inhibitors of free radical formation, including allopurinol and oxypurinol, and spin trapping agents such as *N*-tert-butyl-a-phenylnitrone can reduce reperfusion injury in dog, rabbit and rat heart (10–15).

In this study we have investigated the effects of free radical–generating systems on cardiac function and metabolism in isolated intact hearts and in patch-clamped single heart cells. The results indicate that free radicals markedly impair both glycolytic and oxidative metabolism in heart. These actions may account for some of the functional abnormalities present in reperfused myocardium.

Methods

Intact isolated heart experiments

Preparation. Male New Zealand white rabbits (1.5–2.5 kg) were heparinized and killed by intravenous sodium pentobarbital before thoracotomy. The heart was quickly excised, the septal branch of the left coronary artery was cannulated, and the interventricular septum isolated and mounted in a constant temperature (37°C) nitrogen-filled chamber as described previously (16). A ligature placed at the apex of the septum was tied to a tension transducer and the septum was stimulated at 75 bpm by a bipolar platinum electrode embedded in a corner of the preparation. A perfusion pump (Minipuls 2; Gilson Co., Worthington, OH) maintained a constant flow of 37°C perfusate at a rate of 1.75 ml/min. Standard perfusate consisted of (in millimolar): NaCl, 120; KCl, 4; $CaCl_2$, 1.5; $NaHCO_3$, 25; NaH_2PO_4, 0.44; $MgCl_2$, 1; and dextrose, 5.6. 10 U/liter insulin was added to all perfusates. pH was maintained at 7.3–7.4 by gassing with a mixture of 5% CO_2 and the balance O_2. Venous effluent was collected through a polyethylene tube (PE-90) anchored at the base of the septum and connected to a constant vacuum that deposited the effluent in either an exchangeable glass test tube or polyethylene vials for placement in a gamma counter depending on the protocol. Intracellular potential was monitored with floating glass microelectrodes filled with 3 M KCl (17). Preparations were allowed to equilibrate for 1 h before experimental interventions. Under control conditions this preparation has been shown to maintain stable mechanical and metabolic function for 5–6 h (18, 19).

$^{42}K^+$ washout experiments. Preparations were loaded with perfusate containing $^{42}K^+$ (1.75 μCi/ml) for at least 45 min and then washed out with nonradioactive perfusate for at least 20 min before any intervention. Effluent was collected for 15 s of each 30-s interval during

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1. *Abbreviations used in this paper:* APD, action potential duration; CAT, catalase; FCCP, carbonyl cyanide-*p*-trifluoromethoxyphenylhydrazone; GSS, glycolytic substrates; I-V, current-voltage; MSS, mitochondrial substrates; X, xanthine; XO, xanthine oxidase.

H_2O_2 experiments and for 30 s out of every 60 for all other experiments. Effluent radioactivity was analyzed with a gamma counter (gamma 5500; Beckman Instruments, Fullerton, CA). Counts per minute were corrected for background and decay. By measuring the time constant of $^{42}K^+$ washout under control conditions and using the assumption that $^{42}K^+$ washout and uptake rates are identical during control perfusion (20), the specific activity of the tissue at the start of $^{42}K^+$ washout was calculated. The rate of unidirectional $^{42}K^+$ efflux (in micromoles/minute per gram wet weight) at any time during the washout was then calculated by dividing the effluent counts per minute by the product of the specific activity corrected for that point in time, the effluent collection period and the wet weight of the preparation (20).

Metabolic assays. Venous effluent was collected at 2.5–4-min intervals, frozen, and later analyzed for lactate content. Lactate was determined by a lactate dehydrogenase, NAD assay in which the production of NADH from lactate was monitored spectrophotometrically using a spectrophotometer (model DU-8; Beckman Instruments) operating at 340 nm wavelength (21).

For determination of tissue metabolites, the septum, while still being perfused, was smash frozen between copper blocks precooled in liquid nitrogen and rapidly immersed and stored in liquid nitrogen. A small portion was weighed before and after at least 48 h of drying at 100°C to obtain the dry weight to wet weight ratio. Approximately 200 mg of tissue was extracted with 3 M perchloric acid. Tissue content of creatine phosphate, creatine, ATP, ADP, and AMP was determined using a HPLC system (Waters Associates, Milford, MA) with a C-18 5-μm radial compression cartridge column, 0.3 M $NH_4H_2PO_4$ solvent (pH = 4.0) and an absorbance detector (model 441; Waters Associates) operating at 214 nm wavelength (22).

Single ventricular myocyte experiments

Single ventricular myocytes were isolated enzymatically from the hearts of 300–400-g guinea pigs using the method of Mitra and Morad (23) and studied with the patch clamp technique (24). Whole cell and single channel recordings were made with fire-polished patch electrodes (tip diameter 1–4 μm, resistance 1–3 MΩ) mounted to the headstage of patch-clamp amplifier (model EPC-7; List Electronics, Darmstadt, FRG, or model 1B; Axopatch, Axon Instruments, Burlingame, CA). Data were recorded on a modified videocassette recorder, oscilloscope, and chart recorder and analyzed on an AT style microcomputer. The experimental chamber (0.5 ml) was mounted on the stage of an inverted microscope and was continuously perfused (1–4 ml/min) throughout the experiment. For experiments in which whole cell currents or voltages were measured the standard patch electrode solution dialyzing the cell contained (in millimolar): KCl, 103; KOH, 47; Hepes, 20; EGTA, 14; $CaCl_2$, 1; NaCl, 10; $MgCl_2$, 1; fructose-1,6-diphosphate, 2; sodium pyruvate, 2; NAD, 1; K_2HPO_4, 1, K_2ADP, 0.5; pH 7.1. The latter five substrates were added to allow the myocytes to generate ATP from ADP endogenously (25). The bath solution contained a modified Tyrode's solution consisting of (in millimolar): NaCl, 136; NaOH, 9; NaH_2PO_4, 0.33; KCl, 5.4; $CaCl_2$, 1.8; $MgCl_2$, 1; Hepes, 10; and glucose, 10.

Single-channel currents were recorded from cell-attached patches on permeabilized myocytes. Cells were permeabilized by brief exposure of one end of the cell to a stream of bath solution containing the membrane detergent saponin (0.1%) delivered through a micropipette as described previously (26). In these experiments the standard patch electrode solution contained (in millimolar): KCl, 148; KOH, 2; Hepes, 5; pH 7.3. The bath solution contained (in millimolar): KCl, 140; KOH, 7–10; Hepes, 10; EGTA, 2; $CaCl_2$, 0.5; $MgCl_2$, 2; to which metabolic substrates, free radical–generating systems, or free radical scavengers were added either alone or in combination. Metabolic substrates used were (in millimolar): fructose-1,6-diphosphate, 2; sodium pyruvate, 2; NAD, 1; K_2HPO_4, 1; and K_2ADP, 0.5. All single cell experiments were performed at room temperature (21–23°C).

Free radical–generating systems

Two free radical–generating systems were used: 0.01–1 mM hydrogen peroxide or 1 mM xanthine plus 0.01 U/ml xanthine oxidase. All reagents were obtained from Sigma Chemical Co. (St. Louis, MO). In the presence of agents such as Fe^{2+}, hydrogen peroxide produces hydroxyl free radicals ($\cdot OH$) by either the Fenton or Haber-Weiss reaction (27). Xanthine oxidase catalyzes the reduction of xanthine to uric acid, generating superoxide free radicals ($\cdot O_2^-$) (28) at a rate of $\sim$ 5 μmol/liter/min at these concentrations (8, 29, 30). Superoxide reacts with water to form hydrogen peroxide from which hydroxyl free radicals are generated by the pathways described above. Xanthine oxidase (catalogue item X4500; Sigma Chemical Co.) was chromatographically purified and had an activity of 1–2 U per mg protein. Free radical scavengers included SOD (catalogue item S2515; Sigma Chemical Co.) and catalase (catalogue item C10; Sigma Chemical Co.) both at concentrations of 120 U/ml. SOD catalyzes the conversion of $\cdot O_2^-$ to hydrogen peroxide. CAT prevents $\cdot OH$ formation by degrading hydrogen peroxide to water. The concentrations were chosen based on previous studies demonstrating beneficial effects on free radical–induced cardiac dysfunction (31, 32).

Data analysis

All results are expressed as mean±1 SE in both text and figures. Unpaired *t* tests were used to evaluate statistical significance. $P < 0.05$ was taken to indicate a statistically significant difference.

Results

Effects of free radical–generating systems in intact isolated heart. Fig. 1 illustrates the effects of free radical–generating systems on cardiac function in a typical rabbit septum. Exposure to perfusate containing 1 mM H_2O_2 (*left*) caused an immediate increase in $^{42}K^+$ efflux followed by progressive shortening of the action potential duration (APD). After an initial transient increase, developed tension fell and rest tension increased. Near the end of the 20-min exposure period, developed tension had fallen dramatically concomitant with a marked rise in rest tension. The combination of xanthine and xanthine oxidase (X + XO) caused similar changes in cardiac function as 1 mM H_2O_2 (Fig. 1, *center*). The free radical scavengers SOD and CAT markedly attenuated the effects of X + XO on $^{42}K^+$ efflux, APD and tension (Fig. 1, *right*). In two control preparations, SOD and CAT alone had no effects on cardiac function (not shown).

Fig. 2 summarizes the effects of 1 mM H_2O_2, X + XO, and X + XO in the presence of SOD and CAT, on K^+ efflux rate, APD, developed tension, and rest tension. Lower concentrations of H_2O_2 (0.1 and 0.01 mM) also caused significant increases in $^{42}K^+$ efflux (Fig. 3), but neither caused changes in APD or tension over a 20-min exposure period. Longer exposures (up to 60 min) did cause a decline in rest tension and a decrease in APD (not shown).

The effects of 1 mM H_2O_2 on tissue high energy phosphate content and lactate were also measured. After 2.5 min exposure, at a time when increased $^{42}K^+$ efflux and APD shortening were already occurring, there was no significant change in high energy phosphate concentrations. After 20 min, tissue high energy phosphate levels were significantly depressed, with ATP at 49±11% and creatine phosphate at 46±7% of the control values ($n = 6$, $P < 0.01$). Venous lactate remained at the control level initially and then increased coincident with the elevation in rest tension, reaching 617±165% of control after 20 min ($n = 6$).

Effects of free radical–generating systems on whole cell currents and voltage in single ventricular myocytes. The effects of free radical–generating systems in the rabbit septum are similar to those produced by metabolic inhibitors (33). To

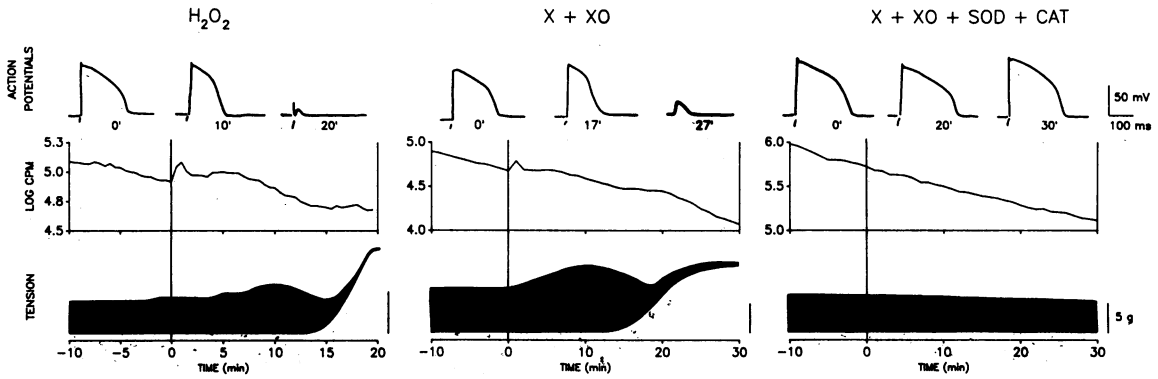

Figure 1. Comparison of the effects of free radical–generating systems on APD, $^{42}K^+$ efflux and tension in typical examples of isolated arterially perfused rabbit intraventricular septa exposed to 1 mM H_2O_2, 1 mM X + 0.01 U/ml XO, or 1 mM X + 0.01 U/ml XO in the presence of 120 U/ml each of SOD and CAT. Temperature, 37°C; heart rate, 75 bpm.

investigate this similarity in greater detail, free radical–generating systems and metabolic inhibitors were compared in single guinea pig ventricular myocytes using the patch clamp technique. Fig. 4 (*left*) shows the combined effects of exposure of a myocyte to the mitochondrial uncoupler carbonyl cyanide-*p*-trifluoromethoxyphenylhydrazone ([FCCP] 0.1 μM) and the glycolytic inhibitor 2-deoxyglucose ([2-DG] 10 mM). These metabolic inhibitors caused progressive APD shortening leading to inexcitability without significant depolarization of the resting membrane potential. Current-voltage (I-V) relations obtained after the APD had shortened to < 25% of control revealed a large outward current reversing near the K^+ equilibrium potential consistent with activation of the ATP-sensitive K^+ current (26). Both H_2O_2 and X + XO (*middle* and *right*) caused similar APD shortening and changes in I-V relations as the metabolic inhibitors, although with a more delayed time course. The mean time required for the APD to shorten to 25% of the control value was 5.9±0.8 min in the presence of metabolic inhibitors (n = 8), 14.5±1.2 min after exposure to H_2O_2 (n = 6), and 35.9±7.5 min after exposure to X + XO (n = 5). Under control conditions, APD also was observed to gradually shorten, reaching 25% of its initial value in 100.9±6.5 min (n = 5). However, the I-V relations were not markedly altered at this point, showing a mild outward shift (Fig. 5, *left*). Inclusion of SOD and CAT (both 120 U/ml) with X + XO in five cells delayed the rate of APD shortening significantly (67.2±8.8 min to 25% control) and prevented the marked alteration in I-V relations (Fig. 5, *right*). Fig. 6 summarizes the times required for the APD to shorten to 25% of the initial control value for each of these conditions. Fig. 7 shows the mean values of the I-V relations under control conditions and after the APD had shortened to < 25% of the initial control value in each case.

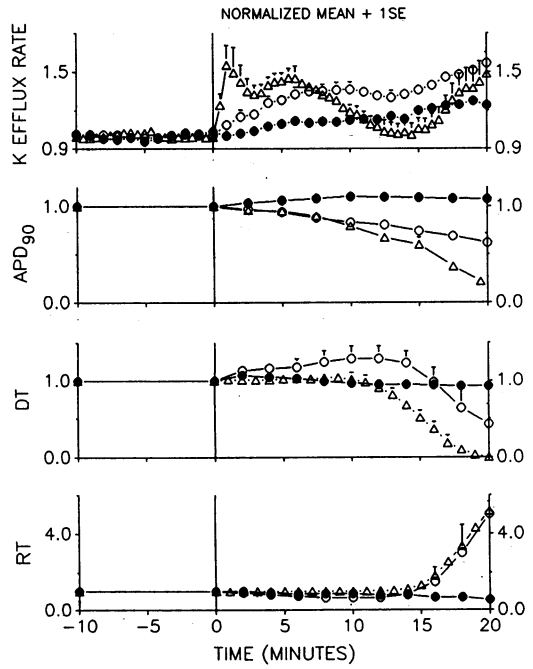

Figure 2. Average data summarizing the effects of the free radical–generating systems on $^{42}K^+$ efflux rate, APD shortening, developed tension (DT) and rest tension (RT) in isolated rabbit septa. After recording control values (−10–0 min), preparations were exposed to either 1 mM H_2O_2 (△; n = 4), 1 mM X + 0.01 U/ml XO (○; n = 3), or X + XO in the presence of 120 U/ml each of SOD and CAT (●; n = 4) for 20 min. Values are mean + SE, normalized to the control values, which were 3.3±0.6 μmol/g per min for $^{42}K^+$ efflux rate, 234±7.5 ms for APD at 90% repolarization (APD_{90}), 7.7±0.7 g for DT, and 2.5±0.3 g for RT. SE bars are shown only if larger than symbols. Temperature, 37°C; heart rate, 75 bpm.

The effects of metabolic inhibitors and free radical–generating systems on inward Ca^{2+} current were also compared. The Ca^{2+} current was isolated by replacing K^+ with Cs^+ in both the bath and patch electrode solutions. The bath solution also contained 10^{-6} M tetrodotoxin and 5 mM Ca^{2+}. In control experiments, raising the tetrodotoxin concentration to 2.5 × 10^{-5} M had no additional effect on the magnitude of the calcium current (n = 4). Both metabolic inhibitors and free radical–generating systems caused a rapid decrease in the am-

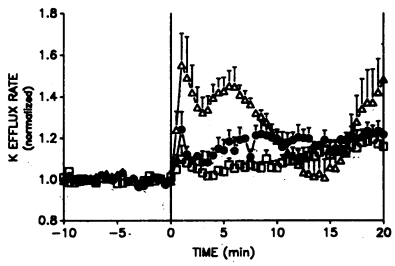

Figure 3. Comparison of the effects of different concentrations of H_2O_2 on $^{42}K^+$ efflux. After recording control $^{42}K^+$ efflux (−10–0 min), preparations were exposed to either 1 mM H_2O_2 (△; n = 5), 0.1 mM H_2O_2 (●; n = 5), or 0.01 mM H_2O_2 (□; n = 4) for a duration of 20 min. Data shown are mean + SE, normalized to the control values of $^{42}K^+$ efflux which averaged 5.9±0.5 μmol/g per min. Temperature, 37°C; heart rate, 75 bpm.

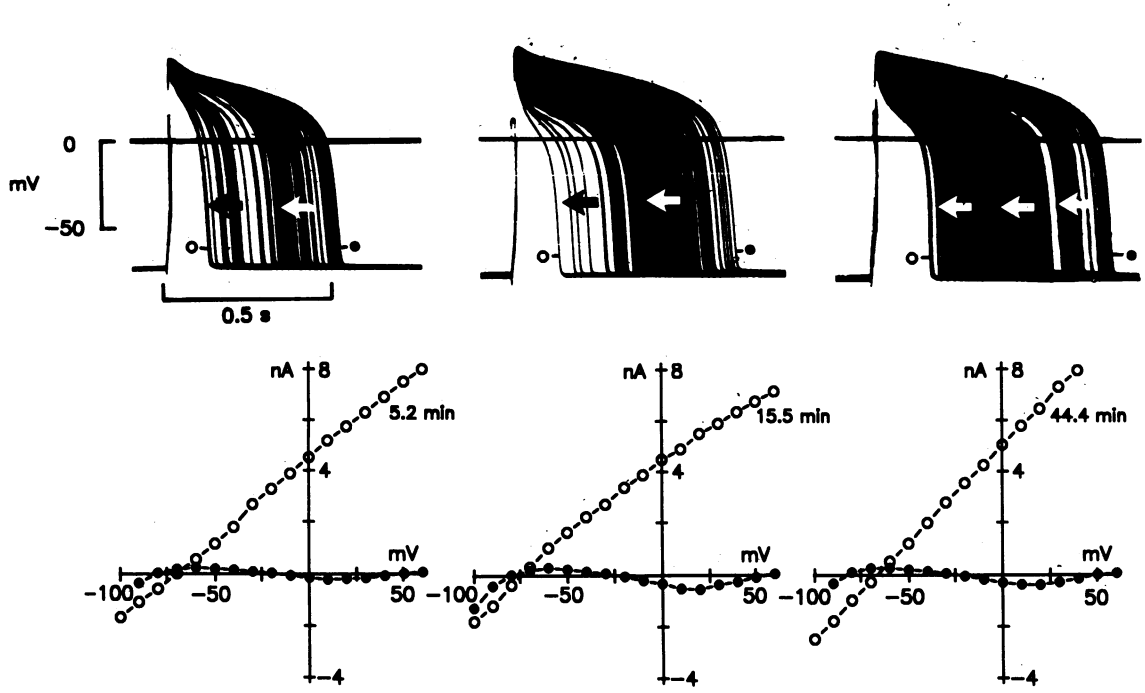

Figure 4. Effect of metabolic inhibitors and free radical–generating systems on the action potential and I-V curves in whole cell voltage-clamped single guinea pig ventricular myocytes. (*Upper traces*) Superimposed action potentials stimulated at 0.2 Hz in three cells during exposure to either: (*left*) 0.1 μM FCCP and 10 mM 2-DG; (*center*) 1 mM H_2O_2; (*right*) 1 mM X + 0.01 U/ml XO. (*Lower graphs*) Control I-V curves (●) and the I-V curves after the APD had shortened to < 25% of the control value at the indicated time (○). Currents are the values at the end of 50-ms voltage clamp pulses from a holding potential of −40 mV.

plitude of the Ca^{2+} current without significantly affecting its voltage dependence (Fig. 8). Fig. 9 summarizes the mean exposure time before $I_{Ca^{2+}}$ declined to 25% of the initial control value during exposure to metabolic inhibitors (15.4±2.5 min, $n = 6$), H_2O_2 (15.0±10.0 min, $n = 6$), X + XO (27.0±1.5 min, $n = 5$) and control conditions (61.5±10.9 min, $n = 6$). When SOD and CAT (each 120 U/ml) were included in the perfusate along with X + XO, the decrease of $I_{Ca}{}^{2+}$ was delayed (43.8±6.2 min, $n = 5$).

Effects of free radical–generating systems on ATP-sensitive K^+ channels. The results in single ventricular myocytes suggest that free radicals may have activated ATP-sensitive K^+ channels by reducing cytosolic ATP, similar to metabolic inhibitors. To test this possibility, single channel current recordings of ATP-sensitive K^+ channels were made in cell-attached patches on myocytes permeabilized at one end with saponin (26). Permeabilized myocytes can generate sufficient ATP endogenously by glycolysis or oxidative phosphorylation to close

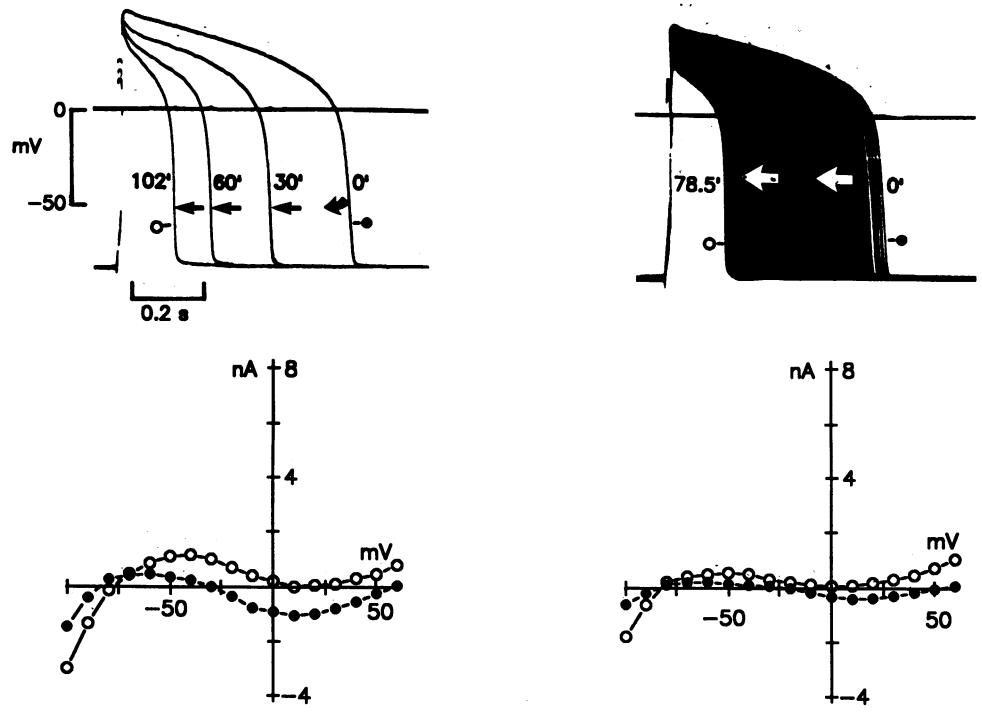

Figure 5. Changes in the action potential and I-V curves in whole cell patch-clamped myocytes under control conditions and during exposure to X + XO with SOD and CAT present. (*Upper traces*) Superimposed action potentials (0.2 Hz) under either control conditions (*left*) or after exposure to 1 mM X and 0.01 U/ml XO in the presence of 120 U/ml each of SOD and CAT (*right*). (*Lower graphs*) Control I-V curves (●) and the I-V curves after the APD had shortened to < 25% of the initial control value (○). Currents are the values at the end of 50-ms voltage clamp pulses from a holding potential of −40 mV.

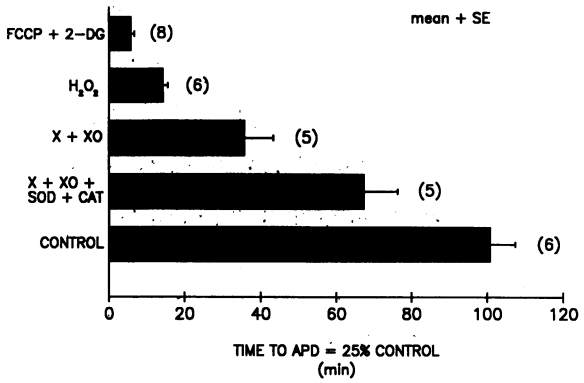

Figure 6. Comparison of average time required for APD to shorten to 25% of the initial control value for the interventions listed in single myocytes under whole cell patch-clamp conditions. Data shown are mean + SE for number of cells indicated in parentheses.

ATP-sensitive K^+ channels when supplied with the appropriate metabolic substrates (25). Fig. 10 shows a typical recording from a permeabilized myocyte in which ATP-sensitive K^+ channels in the cell-attached patch were sequentially activated by removing substrates (*unlabeled arrows*) and then completely suppressed by restoring substrates (*arrows labeled S*) in the bath solution. The substrates included fructose-1,6-diphosphate (2 mM), sodium pyruvate (2 mM), NAD (1 mM), K_2HPO_4 (1 mM), and K_2ADP (0.5 mM) to allow the cell to generate ATP from both glycolysis and oxidative phosphorylation. Activation of ATP-sensitive K^+ channels was always accompanied by shortening of the myocyte which relaxed when substrates were readmitted. In five cells, ATP-sensitive K^+ channels could be activated and suppressed repeatedly by withdrawing and readmitting substrates to the bath until the

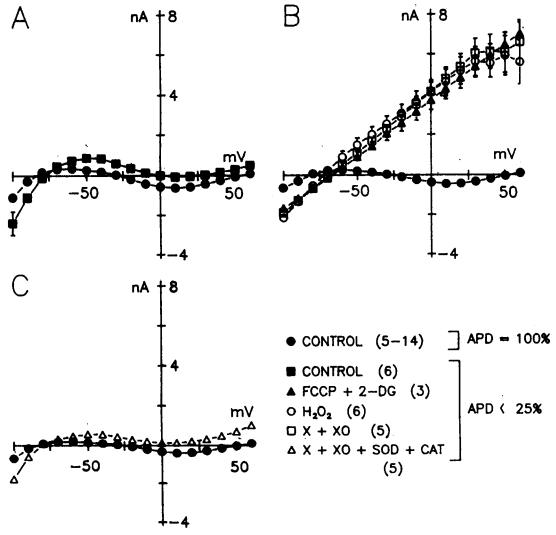

Figure 7. Mean I-V relationships in whole cell patch-clamped single myocytes before and after the APD had shortened to < 25% of the control value. (*A*) Taken under Control conditions. (*B*) Taken during exposure to either 0.1 μM FCCP and 10 mM 2-DG, 1 mM H_2O_2, or 1 mM X + 0.01 U/ml xanthine XO. (*C*) Taken during exposure to X + XO + SOD and CAT. Rate of stimulation was 0.2 Hz. Currents were measured at the end of 50-ms voltage clamp pulses from a holding potential of −40 mV. Values represent the mean±SE for the number of cells indicated in parentheses for each condition. SE bars are shown only if larger than the symbols.

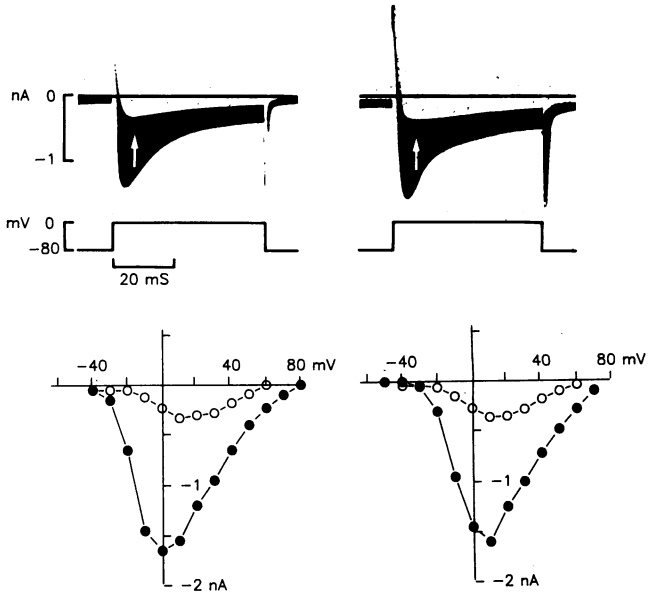

Figure 8. Effect of metabolic inhibitors and H_2O_2 on $I_{Ca}{}^{2+}$ in whole cell patch-clamped single myocytes. (*Upper traces*) Superimposed traces of $I_{Ca}{}^{2+}$ during washing of either 0.1 μM FCCP and 10 mM 2-DG (*left*) or 1 mM H_2O_2 (*right*) during a 50-ms voltage clamp pulse to 0 mV from a holding potential of −80 mV. The lower graphs show the I-V curves before (●) and after (○). Values represent the peak inward current from a holding potential of −80 mV. Cs replaced K^+ in the bath and patch electrode solutions and the bath contained 10^{-6} M tetrodotoxin and 5 mM Ca^{2+}. The voltage protocol activated both low (T) and high (L) threshold calcium channels.

patch ruptured, for nine, eight, seven, seven, and six episodes respectively (mean patch lifetime 45±3.0 min).

Fig. 11 shows that in the presence of either H_2O_2 or X + XO, the same metabolic substrates become ineffective at suppressing ATP-sensitive K^+ channels and reversing the cell shortening. In the top tracing, removal of ATP at the first unlabeled arrow activated ATP-sensitive K^+ channels in the patch. As before, addition of metabolic substrates (*arrow labeled S*) caused the channels to close. 1 mM H_2O_2 was then added to the perfusate and the substrates were removed (*third arrow*), causing the ATP-sensitive K^+ channels to reopen. In the presence of H_2O_2, readdition of the substrates (*fourth*

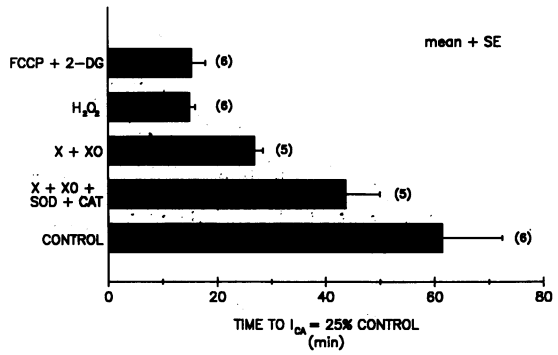

Figure 9. Average time required for peak $I_{Ca}{}^{2+}$ to decrease to 25% of the initial control value in whole cell patch-clamped single myocytes exposed to: 1 mM H_2O_2; 1 mM X and 0.01 U/ml XO; or X + XO plus 120 U/ml each of SOD and CAT. Data shown are mean + SE for the number of cells indicated in parentheses.

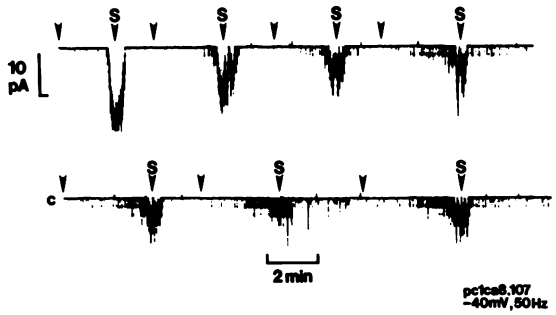

Figure 10. Effect of metabolic substrates on single channel current recordings of ATP-sensitive K^+ channels from a cell-attached patch on a locally permeabilized single guinea pig ventricular myocyte. Inward current is downward, filter setting 50 Hz, tracings are continuous. Patch electrode was held continuously at +40 mV relative to the bath. ATP-sensitive K^+ channels (*downward current deflections*) were repeatedly suppressed when metabolic substrates were added to the bath (*arrows labeled S*) and reopened after modest delay when substrates were removed (*unlabeled arrows*). This sequence was repeated seven times until the patch finally ruptured. See text for description.

arrow) failed to completely suppress the ATP-sensitive K^+ channels, whereas 2 mM ATP remained effective (*fifth arrow*). The effects were reproducible (sixth through ninth arrows). Cell shortening, which accompanied activation of ATP-sensitive K^+ channels, was also reversed by ATP but not by substrates after exposure to H_2O_2 (not shown). After removal of H_2O_2 the substrates remained ineffective at suppressing the channels (*eleventh arrow*) and reversing cell shortening, indicating that the effects were irreversible, at least in the short term. These results were confirmed in five additional permeabilized cells, with substrates becoming ineffective at completely suppressing ATP-sensitive K^+ channels after a mean duration of exposure to H_2O_2 of 5.0±1.3 min. Similar results were obtained with X + XO. As shown in the bottom tracing, metabolic substrates (*fourth arrow*) remained almost completely effective at suppressing ATP-sensitive K^+ channels after the first episode of substrate withdrawal (*third arrow*) in the presence of X + XO, but became ineffective after the

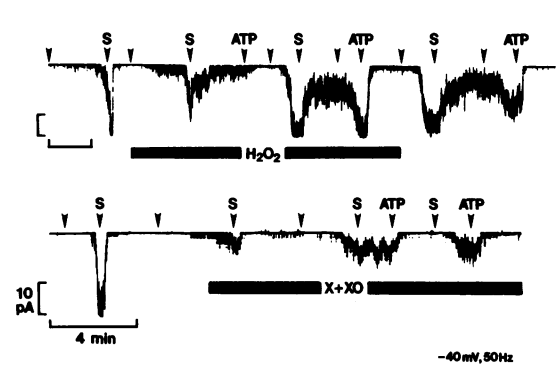

Figure 11. Effect of free radical–generating systems on ability of metabolic substrates to suppress ATP-sensitive K^+ channels recorded from cell-attached patches on permeabilized single guinea pig ventricular myocytes. Conditions are the same as in Fig. 10. Removal of metabolic substrates (*unlabeled arrows*) activated ATP-sensitive K^+ channels which were promptly suppressed by readmitting the substrates (*arrows labeled S*). After exposure to either 1 mM H_2O_2 (*top tracing*) or 1 mM X + 0.01 U/ml XO (*bottom tracing*), metabolic substrates became progressively less effective at closing the channels, whereas 2 mM ATP remained completely effective. See text for further description.

subsequent episodes (*sixth and eighth arrows*). 2 mM ATP remained effective (*seventh and ninth arrows*). In five additional cells, similar results were obtained. Substrates became ineffective at completely suppressing ATP-sensitive K^+ channels after a mean duration of exposure to X + XO of 9.1±1.6 min (in two cells with the first and in four cells with the second episode of substrate withdrawal). The effects of X or XO alone were similar to control.

To determine whether free radical scavengers could prevent or delay the inhibition of metabolism by free radical–generating systems, nine additional permeabilized cells were exposed to X + XO in the presence of SOD and CAT. The free radical scavengers shortened the patch lifetime significantly for unknown reasons, but in seven of the nine cells metabolic substrates remained completely effective at suppressing ATP-sensitive K^+ channels during all the episodes of substrate withdrawal and readmission until the patch ruptured (for four, four, three, three, two, two, and two successive episodes respectively, mean duration of exposure 19.6±1.7 min). Thus the free radical scavengers had at least a partially protective effect.

In additional experiments, the effects of glycolytic versus mitochondrial substrates on ATP-sensitive K^+ channels were compared individually in the presence of 1 mM H_2O_2. In Fig. 12, removal of metabolic substrates from the bath (*first arrow*) caused ATP-sensitive K^+ channels in a cell-attached patch to open. Either mitochondrial substrates (MSS, *second arrow*) or glycolytic substrates (GSS, *fourth arrow*) effectively closed ATP sensitive K^+ channels in the absence of exogenous ATP. Although oxidative phosphorylation was not inhibited during exposure to GSS, we have shown previously that the presence of a mitochondrial inhibitor such as FCCP does not affect the ability of GSS to suppress ATP-sensitive K^+ channels (25). The cell was then exposed to 1 mM H_2O_2 (*middle*), MSS were removed (*first arrow*) and within a short time ATP-sensitive K^+ channels opened. Readdition of either MSS (*second arrow*) or GSS (*third arrow*) was now ineffective at closing the channels in the presence of H_2O_2. However, exogenous ATP (*fourth arrow*) closed the channels. After removal of H_2O_2 (*bottom*), both MSS and GSS remained ineffective at closing the channels. Thus H_2O_2 caused irreversible inhibition of both oxidative phosphorylation and glycolysis without altering the responsiveness of the channels to exogenous 2 mM ATP.

To determine whether the ATP sensitivity of the channels was altered by H_2O_2, the threshold concentration of ATP necessary to suppress ATP-sensitive K^+ channels was compared before and after exposure to H_2O_2. Although the problem of channel rundown made precise determination of the dose response of ATP-sensitive K^+ channel activity to ATP concentration difficult, in three permeabilized cells a reasonable estimate could be obtained in both the absence and presence of 1 mM H_2O_2. ATP concentrations of 0, 0.25, 0.5, 1, and 2 mM were tested. The minimal ATP concentration that produced > 50% suppression of the time-averaged current through ATP-sensitive K^+ channels in the patch (relative to the average current in the absence of ATP) was 0.5, 0.5, and 1 mM, respectively, in the three cells before exposure to H_2O_2, and 0.25 mM, 0.5, and 1 mM with H_2O_2 present. Thus, H_2O_2 did not decrease the sensitivity of ATP-sensitive K^+ channels to ATP.

Discussion

Mechanism of free radical–induced changes in cardiac function. The mechanisms whereby oxygen free radicals contribute

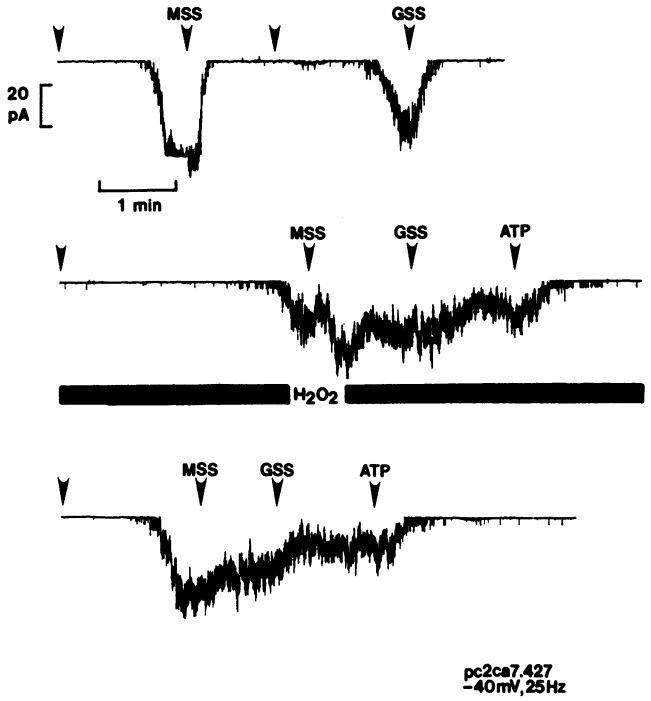

Figure 12. Effect of H_2O_2 on ability of glycolytic versus mitochondrial metabolic substrates to suppress ATP-sensitive K^+ channels recorded from a cell-attached patch on a permeabilized single guinea pig ventricular myocyte. Same conditions as in Figs. 10 and 11 except that individual rather than combined metabolic substrates for glycolysis (GSS) or mitochondrial oxidative phosphorylation (MSS) were used. GSS consisted of 2 mM fructose-1,6-diphosphate, 1 mM NAD and $K_2H_2PO_4$, and 0.5 mM ADP. MSS consisted of 2 mM pyruvate, glutamate, and creatine, 1 mM $K_2H_2PO_4$, and 0.5 mM ADP. In the *top trace,* either GSS or MSS was effective at suppressing ATP-sensitive K^+ channels that opened when metabolic substrates were removed from the bath (*unlabeled arrows*). During (*middle trace*) or after (*bottom trace*) exposure to 1 mM H_2O_2, addition of GSS or MSS failed to suppress the channels, although 2 mM ATP remained effective. See text for description.

to cardiac dysfunction associated with ischemia, postischemic myocardial stunning and postischemic reperfusion injury are not fully understood. Biochemically, free radicals are thought to form lipid peroxides that deplete phospholipids from cell membranes, inhibit membrane-associated enzymes, and interact with proteins to cause tissue damage (34–37). Specific targets that are damaged by free radical–induced lipid peroxides include mitochondria, Na^+-K^+ ATPase, myofibrillar ATPase, and sarcoplasmic reticulum (6–9). Free radicals may also damage vascular endothelium (31), secondarily affecting cardiac function by impairing blood flow.

In the arterially perfused rabbit interventricular septum, free radical–generating systems caused marked cellular K^+ loss and APD shortening followed by a fall in developed tension, increased rest tension, and depression of high energy phosphate levels. The time course of these effects varied depending on the free radical–generating system used, probably as a result of different rates of production of free radical species with the different systems. The effects of free radical–generating systems were qualitatively similar to those occurring during metabolic inhibition in the septal preparation. The immediate increase in K^+ efflux when total cellular high energy phosphates and contractile function were still normal is characteristic of inhibition of glycolysis (33). Increased K^+ efflux may have been caused by activation of ATP-sensitive K^+ channels which have been shown to be preferentially dependent on ATP derived from glycolysis (25). Glycolytic enzymes associated with these channels may be particularly susceptible to damage by extracellular free radicals because of their proximity to the sarcolemma. We did not find any evidence that free radicals directly altered the sensitivity of ATP-sensitive K^+ channels to ATP. After its initial increase K^+ efflux often decreased transiently, consistent with progressive APD shortening, which reduced the time-averaged driving force for K^+ efflux.

Contractile dysfunction and reduction in total cellular high energy phosphate levels induced by free radical–generating systems developed after a delay consistent with gradual progressive inhibition of oxidative metabolism, probably resulting from cumulative intracellular penetration of free radicals. The secondary increase in K^+ efflux rate during this phase seen with X + XO and the highest concentration of H_2O_2 may reflect the increasing severity of combined inhibition of glycolysis and oxidative phosphorylation. The findings in permeabilized single ventricular myocytes demonstrated that H_2O_2 and X + XO directly impaired both glycolytic and oxidative (mitochondrial) ATP production. In nonpermeabilized myocytes the effects of free radical-generating systems and metabolic inhibitors were also very similar. Both caused marked action potential shortening, activation of the ATP-sensitive K^+ current, depression of $I_{Ca}{}^{2+}$ and cell shortening. The time course of these changes was more rapid during exposure to metabolic inhibitors than during exposure to free radical–generating systems indicating that the effects of free radicals on metabolism take some time to develop. The dose and free radical species generated are also important as the time course was different for X + XO and various concentrations of H_2O_2. Deleterious effects of free radicals on mitochondrial function and myocardial substrate oxidation have been noted previously (29, 38, 39) but direct effects on anaerobic glycolysis have not been described to our knowledge.

Although free radical–induced impairment of metabolism could account at least qualitatively for all of the observed abnormalities in cardiac function, free radical–induced damage to other cellular organelles may also have been important. Free radicals have been shown to impair calcium sequestration by the sarcoplasmic reticulum, and calcium overload has been implicated in the pathogenesis of free radical–induced damage (6, 39–42). In the septum, the transient increase followed by a decrease in developed tension and rise in rest tension are consistent with a progressive rise in cytosolic calcium. Other investigators have shown that the delayed increase in rest tension in septa exposed to H_2O_2 is accompanied by increased $^{47}Ca^{2+}$ uptake (43). However, abnormal calcium handling is also a feature of metabolic inhibition, although its precise relationship to contractile dysfunction is controversial (44). Calcium overload may itself cause metabolic impairment by inhibiting mitochondrial ATP production (9). We believe that calcium overload is unlikely to have been an important cause of metabolic inhibition in the single cells, because the solutions bathing the cytoplasm were highly buffered with EGTA, which minimized any fluctuations in cytosolic calcium. This also eliminated our ability to assess whether a rise in intracellular calcium occurred in the single cells. However, $I_{Ca}{}^{2+}$ decreased during exposure to free radical–generating systems, presumably because of reduced channel phosphorylation due to inhibition of metabolism (although a direct effect cannot be excluded). Thus calcium influx through low (T) or high (L)

threshold calcium channels could not be the cause of calcium overload during exposure to free radical–generating systems.

It is unlikely that contaminants in the free radical–generating systems were responsible for the deleterious effects on cardiac function in our experiments. Both H_2O_2 and X + XO had very similar effects on electromechanical and metabolic function although the time courses differed somewhat. The effectiveness of free radical scavengers at preventing or delaying these effects also suggests that free radicals rather than contaminants were the cause of the functional abnormalities. We did not attempt to determine which free radical species were specifically responsible for the changes we observed. The observation that H_2O_2 (a source of hydroxyl radical but not superoxide) caused effects similar to X + XO (a source of superoxide anion, H_2O_2 and hydroxyl radical) suggests that superoxide anion was not a requirement for the observed effects. Biochemically, the hydroxyl radical is one of the most cytotoxic oxygen metabolites and is not scavenged directly by any specific cellular defense mechanism (3). Its formation may be the final common pathway leading to lipid peroxidation and other damaging effects of free radicals, although direct toxicity from H_2O_2 on metabolism cannot be excluded.

Relevance to myocardial ischemia and reperfusion injury. It remains speculative whether the concentrations of free radicals used in this study are comparable to those occurring during ischemia and reperfusion. In normally perfused heart ~ 5% of oxygen consumed is univalently reduced to form superoxide anion (45–47). Ubiquitous endogenous free radical scavengers such as SOD, CAT, and glutathione peroxidase normally prevent superoxide and H_2O_2 from reaching toxic levels. During ischemia, however, endogenous free radical scavenger activity decreases and substrates for free radical production become elevated (48, 49). Using EPR spectroscopy, total tissue concentrations of free radicals in ischemic rabbit heart in vitro reached ~ 3 μM, and up to 20 μM transiently during reperfusion (4, 50). Although EPR cannot distinguish intracellular from extracellular free radicals, it is presumed that intracellular free radical levels were significantly elevated under these conditions. The contribution of extracellular free radicals to reperfusion injury is uncertain and in vivo, intracellular penetration of exogenous free radicals may be significantly limited by plasma proteins and antioxidant systems (51). On the other hand, in the septum concentrations of H_2O_2 as low as 10 μM applied extracellularly caused detectable abnormalities in sarcolemmal function such as increased K^+ efflux. In single cells, intracellular penetration of free radicals was undoubtedly facilitated by permeabilization of the membrane with saponin. The nonpermeabilized single cells may also have been sensitized to the effects of exogenous free radicals because dialysis of the cytoplasm by the patch electrode solution may have washed out some endogenous free radical scavengers and metabolic enzymes.

Despite these limitations, there are notable similarities in cardiac dysfunction in reperfused myocardium and myocardium exposed to free radical–generating systems including persistent cellular K^+ loss, depressed tension development, elevated rest tension and persistently reduced levels of high energy phosphates. In reperfused heart these abnormalities may be irreversible, or prolonged but eventually reversible (so-called "stunned" myocardium). In single ventricular myocytes exposed to free radical–generating systems, inhibition of glycolytic and oxidative metabolism was irreversible over the short time span of these experiments. In the septum, the effects of free radical–generating systems also appeared to be irreversible. However, this does not exclude the possibility that in vivo over a prolonged period of hours or days analogous to the time course of recovery of stunned myocardium, reparative processes in the heart might be able to restore normal metabolic function after exposure to free radical–generating systems.

In addition to contractile dysfunction in reperfused myocardium, free radicals have also been implicated in the genesis of reperfusion arrhythmias based on the observation that free radical scavengers decrease the frequency of reperfusion arrhythmias in isolated heart preparations (52–55). Free radical–generating systems caused an increase in K^+ efflux and APD shortening in the isolated rabbit septum and activated ATP-dependent K^+ currents and decreased $I_{Ca^{2+}}$ in single ventricular myocytes. These electrophysiological abnormalities may contribute to the electrical instability of reperfused myocardium.

Our observations suggest that a major action of free radicals during ischemia and reperfusion may be prolonged and perhaps irreversible inhibition of glycolysis and oxidative metabolism. The use of free radical scavengers may lead to improved ventricular function in models of cardiac reperfusion by preserving myocardial metabolism and thus providing cellular repair mechanisms with adequate reserves of high energy phosphates.

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