Coronary Artery Contractility, Na^+-Pump and Oxygen Radicals

A. ELMOSELHI, A. BUTCHER, S. E. SAMSON and A. K. GROVER

Department of Biomedical Sciences, McMaster University
1200 Main Street West, Hamilton, Ontario, Canada L8N 3Z5

Abstract. Oxygen radicals accumulated during ischemia and reperfusion may affect coronary contractility by endothelium dependent and independent pathways one of which may involve Na^+-pump. Here we report a contractility assay for Na^+-pump in pig coronary artery and use it to examine the effects of hydrogen peroxide and superoxide. Coronary artery rings contracted in a K^+-free Krebs solution and relaxed upon subsequent exposure to K^+. The relaxation approximated a single exponential decay whose rate constant depended on $[K^+]^2$. This K^+-induced relaxation was abolished by ouabain and was attributed to Na^+-pump. In tissues pretreated with peroxide, the rate of relaxation of the K^+-free contracted arteries decreased with an $IC_{50} = 1.6 \pm 0.6$ mmol/l for peroxide. Another set of tissues was pretreated with the superoxide generating system containing 0.3 mmol/l xanthine + varying concentrations of xanthine oxidase (XO) and precontracted in K^+-free Krebs solution. The rate of the K^+-induced relaxation decreased with $IC_{50} = 24 \pm 8$ mU/ml for XO. Thus, using the relaxation assay we conclude that exposing coronary arteries to oxygen radicals can damage Na^+-pumps.

Key words: Free radicals — Myocardium — Ischemia — ATPase — Vascular smooth muscle — Potassium.

Introduction

Accumulation of H^+ and various reactive oxygen species such as peroxides, superoxide, hydroxyl radicals and hypochlorites occurs during cardiac ischemia and reperfusion (Zweier, 1988; Downey 1990; Bernofsky 1991). The reactive oxygen may interact directly with proteins or cause lipid peroxidation and the modified lipids may influence the activities of the various proteins. The effects of such alterations in the protein activities can compromise cardiovascular function. In cardiac

Correspondence to: Dr. Ashok Grover, Department of Biomedical Sciences, McMaster University, 1200 Main Street West, Hamilton, Ontario L8N 3Z5 Canada

myocytes, reactive oxygen has been reported to act on Ca^{2+} pumps, Ca^{2+} channels, Na^+-H^+-exchange, Na^+-pump, contractile proteins and the mitochondrial protein pyruvate dehydrogenase (Kramer et al. 1984; Kim and Akera 1987; Kaneko et al. 1989, 1991; Dixon et al. 1990; Kukreja et al. 1990; Xie et al. 1990; Vlessis et al. 1991; Kirshenbaum and Singal 1992). However, less is known about the effects of these agents on the coronary artery (Grover and Samson 1988, 1989; Roberts et al. 1990; Grover et al. 1992; Samaha et al. 1992; Todoki et al. 1992; Elmoselhi et al. 1994). In biochemical and transport experiments peroxide and superoxide can damage the Na^+-pumps (Elmoselhi et al. 1994). In order to test how the damage to the Na^+-pump relates to the artery contractility, we developed a contractility based assay of the pump and tested the effects of peroxide and superoxide in this assay.

Materials and Methods

Xanthine oxidase

Xanthine oxidase was purified from butter milk according to Rajagopalan (1985). Immediately before each use the conversion of xanthine to uric acid was monitored as described previously (Grover and Samson 1987). An enzyme milliunit (mU) was defined as one thousandth of the amount of enzyme required to convert 1 μmol of xanthine to uric acid in 1 min at 37 °C.

Relaxation assay

Pig hearts were obtained from slaughter house and placed on ice. Within 1 h the tissue slices containing the left coronary artery were placed in Krebs solution bubbled with 95% O_2 and 5% CO_2 and dissected further to obtain 2–3 mm long pieces of coronary artery. The Krebs solution contained the following in mmol/l: 115 NaCl, 5 KCl, 22 $NaHCO_3$, 1.7 $CaCl_2$, 1.1 $MgCl_2$, 1.1 KH_2PO_4, 0.038 EDTA and 7.7 glucose. The tissues obtained were devoid of cardiac muscle cells and denuded of endothelium as described previously (Grover et al. 1987). 2–3 mm long pieces of the denuded coronary artery rings were mounted in muscle baths at 37 °C under basal tension of 6 g. The tissues were allowed to equilibrate for 60 min in normal Krebs solution and then tested for their ability to contract to 60 mmol/l KCl at the beginning of each experiment. The tissues were then washed 4 times in K^+-free Krebs solution (KCl replaced with NaCl) in which these contracted typically for 1 h, after which aliquots of 2 mol/l KCl were added to attain the specified KCl concentrations and the resulting relaxation was monitored (Grover et al. 1980). The tissues were again washed in normal Krebs solution and contraction to 60 mmol/l KCl was monitored at the end of each experiment. In the experiments using peroxide the tissues were placed in K^+-free Krebs solution and 2 min later peroxide was added to attain the specified final concentration. Contracture in K^+-free Krebs solution was allowed for 2 h before monitoring the K^+-induced relaxation. Since superoxide is known to relax the coronary artery to examine the effect of long term treatment with superoxide the tissues were washed 3 times in K^+-free Krebs solution placed 0.3 mmol/l xanthine dissolved in K^+-free Krebs solution, to which specified concentrations of xanthine oxidase were added after 1 h the tissues were washed in normal Krebs solution to remove the added xanthine

and xanthine oxidase and incubated for another 2 h in K^+-free Kreb's solution before monitoring the K^+-induced relaxation.

Data analysis

The relaxation data were analyzed as single exponentials. Where indicated the data were fitted by hyperbolic kinetics to determine EC_{50} or IC_{50} values. For $[K^+]$-dependence, the data were analyzed against $[K^+]^2$. All the curve fitting was carried out on unweighted data using FigP software (Biosoft). All the values are mean ± SEM using n animals. Student's unpaired 2-tailed t-test was used to test each null hypotheses and the p values of > 0.05 were considered to negate it.

Chemicals

Xanthine, superoxide dismutase, ouabain and catalase were purchased from Sigma Chemical Company and hydrogen peroxide from BDH Chemicals. All other chemicals were purchased from standard commercial sources.

Results

Characterization of K^+-relaxation assay

It has been reported that smooth muscle tissues contract when placed in K^+-free solution and that adding K^+-back results in relaxation which depends on Na^+-pump (Grover et al. 1980; Rangachari et al. 1983). The first objective was to develop this assay for the pig coronary artery. The pig coronary artery rings contracted when placed in K^+-free Krebs solution (Fig. 1*A*). In initial experiments, it was determined that the optimal basal tension was 3–6 g and that the contraction reached a near maximum in 30 to 40 min. The maximum contraction obtained in K^+-free Krebs solution was 2.6 ± 0.3 N/g (mean ± S.E.M, $n = 19$). This value corresponded to $32 \pm 4\%$ of the maximum contraction of 8.1 ± 0.3 N/g obtained upon membrane depolarization with 60 mmol/l KCl. After the K^+-free contracture, adding 2 mmol/l KCl relaxed the arteries rapidly returning their tone to the precontraction level within several min (Fig. 1*A*). The contraction remaining after adding KCl was analyzed as a single exponential against time taking the time of addition of KCl as zero (Fig. 1*C*). However, if 1 mmol/l ouabain was added prior to adding KCl, no relaxation was observed (Fig. 1*B*).

Since the magnitude of relaxation after adding KCl merely reflects a difference between two steady states, the rate of relaxation, which is a kinetic parameter, was used as an indication of the initial velocity of Na^+-pump in all the further experiments. The rate of the K^+-induced relaxation depended on its concentration (Fig. 1*D*). Since 2 K^+ are expected to be transported per cycle (Apell 1989), a hyperbolic relationship would be expected between the Na^+-pump activity and $[K^+]^2$. Therefore, the data in Fig. 1*D* were fitted into this curve. The best fit gave a $K_{0.5}$ value of 0.72 ± 0.17 mmol/l.

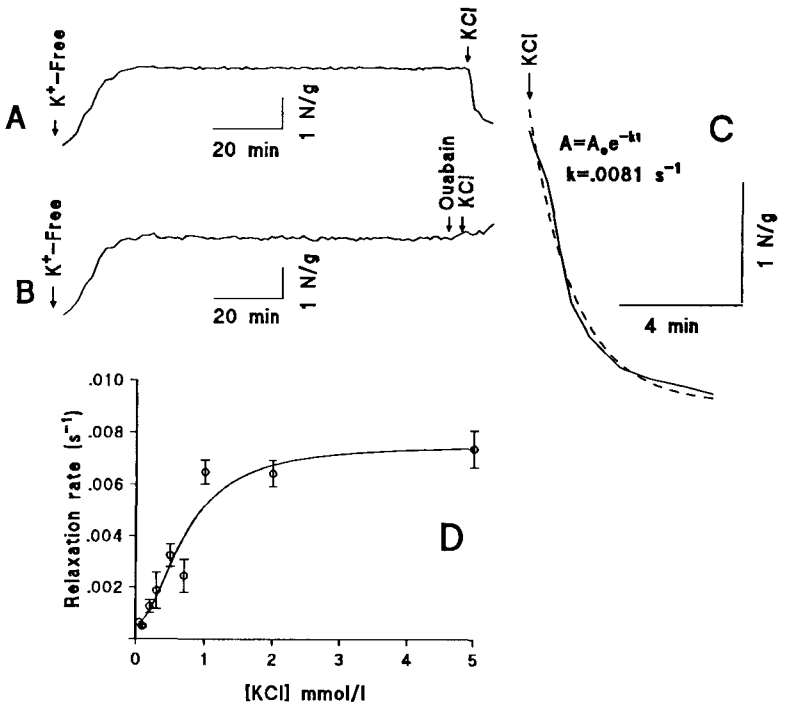

Figure 1. K^+-induced relaxation of K^+-free contracted coronary artery rings. *A*. Coronary artery rings were precontracted in K^+-free solution. Adding 2 mmol/l KCl caused a relaxation. *B*. As in *A* except that 1 mmol/l ouabain was added before adding KCl. *C*. The relaxation with 2 mmol/l KCl in *A* was analyzed as a single exponential to obtain the rate constant k. Solid line represents the data and the dotted line is the exponential fitted curve. *D*. $[K^+]$-dependence of the rate constants for the K^+-induced relaxation. The protocol similar to that in *A* was used. The relaxation rates calculated as in *C* depended on $[KCl]^2$ with a $K_{0.5}$ of 0.72 ± 0.17 mmol/l. The values shown are mean ± S.E.M with tissues from 3–6 animals at each [KCl].

Effect of peroxide on K^+-relaxation

For examining the effect of peroxide on the K^+-induced relaxation, the tissues were precontracted for 2h in the K^+-free Krebs solution containing peroxide and then KCl was added (Fig. 2*A*). The resulting relaxation could be described as a single exponent (Fig. 2*B*). The peroxide treatment caused a decrease in the relaxation rate with an IC_{50} value of 1.6 ± 0.6 mmol/l when 2 mmol/l KCl was used for the relaxation step and with an IC_{50} value of 2.3 ± 0.4 mmol/l when 5 mmol/l KCl was used (Fig. 2). The two IC_{50} values for peroxide using 2 or 5 mmol/l KCl in the relaxation assay were not significantly different ($p > 0.05$).

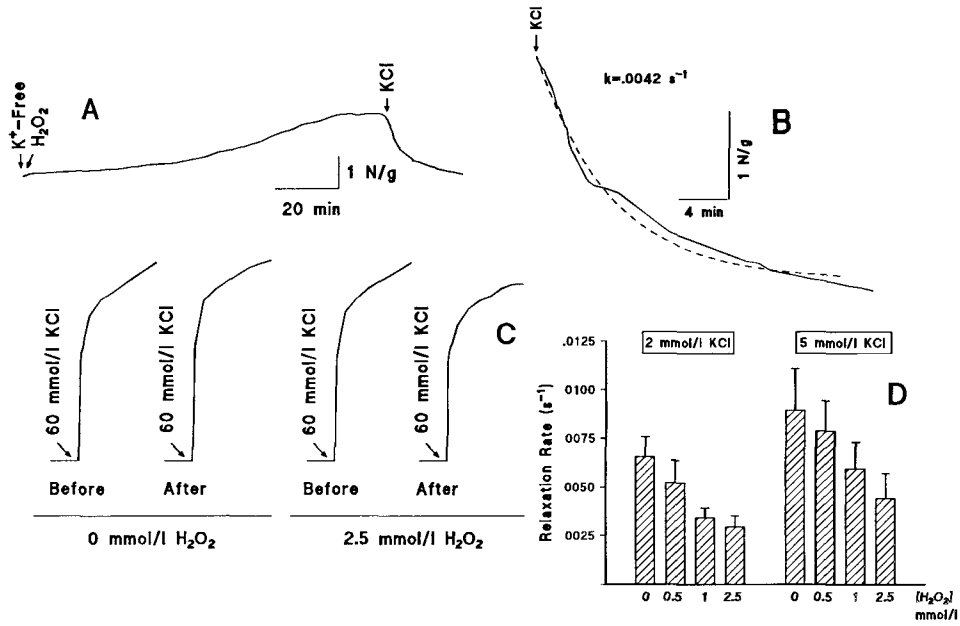

Figure 2. Effect of peroxide on K^+-relaxation. *A*. Typical tracing for peroxide treatment. The tissues were placed in K^+-free solution and 2 min later H_2O_2 was added to 2.5 mmol/l. After 2 h incubation, the relaxation with 2 mmol/l KCl was monitored. *B*. The relaxation to 2 mmol/l KCl in *A* was analyzed as a single exponential to obtain the rate constant k. Solid line represents the data and the dotted line is the exponential fitted curve. *C*. Contraction to 60 mmol/l KCl. "Before" represents the contraction to 60 mmol/l KCl at the beginning of the experiment and "After" is the KCl contraction at the end. In between the treatment with the specified concentration of peroxide, K^+-free contracture and the K^+-relaxation were examined. *D*. $[H_2O_2]$-dependence: H_2O_2 was added to the K^+-free Krebs solution to attain the specified concentrations. After an incubation for another 2 h, 2 or 5 mmol/l KCl was added. The values of the rate constant shown at each point are mean ± S.E.M of 3–12 animals. When 2 mmol/l KCl was used, the rate constant decayed with an IC_{50} value of 1.6 ± 0.6 mmol/l for peroxide and when 5 mmol/l KCl was used, the IC_{50} value was 2.3 ± 0.4 mmol/l. The two values were not statistically different ($p > 0.05$).

The following control experiments were also performed to examine the various effects of peroxide:

a) In experiments where tissues were treated with 2.5 mmol/l peroxide with or without superoxide dismutase (20 units/ml), superoxide dismutase did not significantly affect the inhibition of the relaxation by 2.5 mmol/l peroxide indicating that peroxide did not switch on a cellular pathway causing production of superoxide which in turn reacted with the Na^+-pump was unlikely.

b) Basal tone of the tissues was monitored with and without 2.5 mmol/l peroxide for 1 h in normal Krebs solution. The observed change in basal tone was 0.09 ± 0.03 N/g (mean ± S.E.M, $n = 3$) in control and 0.16 ± 0.07 N/g ($n = 3$) in the peroxide treated tissues. Thus the peroxide treatment did not cause a significant increase in the basal tone ($p > 0.05$).

c) The peroxide treatment delayed the onset of the contracture in K^+-free solution (Fig. 2*A*) but the maximum force generated due to the K^+-free contracture in the tissues pretreated with 2.5 mmol/l H_2O_2 was 3.1 ± 0.7 N/g and did not differ significantly ($p > 0.05$) from the force generated by the untreated tissues (2.6 ± 0.3 N/g).

d) The tension developed with 60 mmol/l KCl was monitored in the tissues before and after each experiment and the second contraction expressed as percent of the first contraction. In the control tissues the second KCl contraction was $111 \pm 4\%$ ($n = 19$) of the first. The second contractions in the tissues pretreated

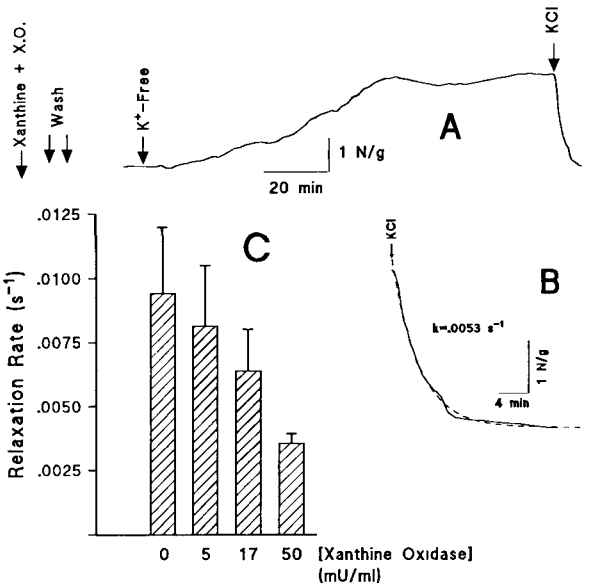

Figure 3. Effect of superoxide on K^+-induced relaxation. *A*. The tissues were pretreated for 1 h with 0.3 mmol/l xanthine was added along with K^+-free solution and 17 mU/ml of X.O. and then washed in Krebs solution. K^+-free Krebs without xanthine or xanthine oxidase was then added to obtain a contraction and 2 mmol/l KCl added to monitor the relaxation. *B*. The relaxation with 2 mmol/l KCl in *A* was analyzed as a single exponential to obtain the rate constant k. Solid line represents the data and the dotted line is the exponential fitted curve. *C*. The coronary artery rings were treated according to the protocol in *A* but with different concentrations of xanthine oxidase, and analyzed as in *B*. The relaxation rate values are mean ± S.E.M of tissues from 2–3 animals at each point. These values correspond to IC_{50} value of 24 ± 8 mU/ml for xanthine oxidase.

with 1 mmol/l peroxide (96 ± 7 % ($n = 5$)) were not significantly different from the control but those in the tissues pretreated with 2.5 mmol/l peroxide (77 ± 14% ($n = 3$)) were marginally lower ($p < 0.05$). The contractions developed by 60 mmol/l KCl were fast in control tissues and those treated with peroxide (Fig. 1*A* and 2*C*).

Effect of superoxide on K^+-relaxation

In order to examine the effect of superoxide, the tissues were pretreated for 1 h in superoxide by incubation in K^+-free Krebs solution + 0.3 mmol/l xanthine + specified concentrations of xanthine oxidase. Then they were washed in normal Krebs solution and allowed to develop another contracture in K^+-free Krebs solution. 2 mmol/l KCl was added after exposure to K^+-free solution and the resulting relaxation was monitored (Fig. 3*A*). This relaxation could be described as a single exponent (Fig. 3*B*). The superoxide pretreatment decreased the rate of the K^+-relaxation with an IC_{50} value of 24 ± 8 mU/ml for xanthine oxidase (Fig. 3*C*).

The following control parameters were also monitored. Contractions to 60 mmol/l KCl, monitored before and after the superoxide treatment, were rapid. Expressed as percent of the first contraction in each tissue, the second contractions in the tissues treated with 0, 1.7, 5, 16.7 and 50 mU/ml of X.O. were 111 ± 4 ($n = 19$), 108 ± 6 ($n = 2$), 84 ± 9 ($n = 3$), 94 ± 13 ($n = 3$) and 91 ± 9 ($n = 4$)%, respectively. Thus the superoxide pretreatment did not significantly alter the contraction due to membrane depolarization by 60 mmol/l KCl. It also did not cause an increase in the basal tone. In tissues pretreated with 50 mU/ml xanthine oxidase, the contraction to K^+-free developed slowly (Fig. 3*A*) but the final value of the force (2.9 ± 0.5 N/g ($n = 3$)) did not differ significantly ($p > 0.05$) from the untreated tissues (2.6 ± 0.3 N/g). The magnitude of relaxation on adding 2 mmol/l KCl after the K^+-free contracture also did not alter significantly upon superoxide treatment. For instance the relaxation in the control tissue was 96 ± 4% ($n = 12$) of the total contraction and in tissues treated with 50 mU/ml of X.O., it was 95 ± 3% ($n = 4$).

Discussion

The results show that (a) the KCl relaxation of the denuded pig coronary artery rings previously exposed to K^+-free solution could be described as a single exponent, (b) the rate constant for this relaxation depended on $[K^+]^2$, (c) the relaxation was abolished by ouabain, and (d) exposing the tissues to peroxide or superoxide reduced the rate of relaxation consistent with a damage to the Na^+-pump. The Discussion will focus on the nature of the assay used, comparison of the effects of reactive oxygen observed here with those reported in the literature, and the implications of these findings in coronary ischemia.

It has been shown in other smooth muscles that exposing tissues to K^+-free solutions causes a membrane depolarization and a concomitant contraction and adding K^+ back repolarizes the membranes and relaxes the tissues. The validity of this assay in pig coronary arteries and its usefulness in examining the effects of free radicals on the Na^+-pump activity are confirmed by the following observations:

a) There was a hyperbolic relationship between the rate constants and $[K^+]^2$. This is consistent with the stoichiometry of the Na^+-pump in that 2 K^+ are transported per cycle (Apell 1989);

b) Ouabain, which is a selective inhibitor of the Na^+-pump, inhibited the K^+-relaxation;

c) Peroxide and superoxide inhibited the Na^+-pump in the relaxation assay. However, these agents also slowed the development of contracture in K^+- free solution without influencing the final magnitude of the contracture but the contraction to 60 mmol/l K^+ in tissues treated with either agent remained rapid. Thus the reduction in the speed of contracture was not due to a general tissue damage and may also be associated with the inhibition of the Na^+-pump. However, a contribution from a damage to the K^+-channels in delaying the onset of this contracture or indirect pathways involving neuronal cells, can not be ruled out; and

d) A general increase in membrane permeability to ions such as Na^+ and Ca^{2+} is unlikely because pretreatment with neither agent led to significant changes in basal tone as would be expected if the cells were to become leaky to these ions.

The effect of peroxide was not blocked by superoxide dismutase and that of superoxide was not inhibited by catalase. Thus it is unlikely that the observed results are due to one free radical switching on a pathway which caused production of the other which in turn caused the damage but the possibility exists of the formation of other free radicals or lipid peroxides which may indirectly affect the Na^+-pump in the smooth muscle or neuronal cells in the tissue.

In the pig coronary artery, the effect of peroxide and superoxide on Na^+- and Ca^{2+}-pumps in the isolated membranes and on Rb^+-uptake by artery rings have been reported previously (Grover and Samson 1988, 1989; Grover et al. 1992; Elmoselhi et al. 1994) but similar detailed information on the effect on other activities is not available. Ouabain sensitive Rb^+-uptake by the artery rings and Ca^{2+}-uptake in the isolated sarcoplasmic reticulum Ca^{2+} pump are extremely sensitive to peroxide and superoxide but the plasma membrane Ca^{2+}-pump and the hydrolysis reaction of the Na^+-pump are less sensitive. In studies on ischemia, a number of reactive oxygen species have been reported to be produced (Zweier 1988; Downey 1990; Bernofsky 1991). Consistent with a damage to the Na^+-pump in rat ventricles, the Na^+ content of the cells is increased during ischemia and reperfusion injury (Kirshenbaum and Singal 1992). It is anticipated that such damage to coronary arteries would decrease their pliability during ischemic injury.

Acknowledgements. The authors thank Drs P K Rangachari and M Muller for their critical comments and T Roketta and G Kritsch for expert secretarial help This work was supported by a Grant-in Aid and a Career Investigator Award to A K G from Heart and Stroke Foundation of Ontario

References

Apell H -J (1989) Electrogenic properties of the Na,K pump J Membrane Biol **110,** 103—114

Bernofsky C (1991) Nucleotide chloramines and neutrophil-mediated cytotoxicity FASEB J **5,** 295—300

Dixon I M C , Kaneko M Hata T , Panagia V , Dhalla N S (1990) Alterations in cardiac membrane Ca^{2+} transport during oxidative stress Mol Cell Biochem **99,** 125—133

Downey J M (1990) Free radicals and their involvement during long term myocardial ischemia and reperfusion Annu Rev Physiol **52,** 487—504

Elmoselhi A , Butcher A , Samson S E , Grover A K (1994) Free radicals uncouple the sodium pump in pig coronary artery Amer J Physiol **266** (Cell Physiol 35), C720—C728

Grover A K , Samson S E (1987) Impurities in commercial xanthine oxidase inhibit Ca-pump and interfere in contractility of pig coronary artery Biochem Biophys Res Commun **143,** 575—581

Grover A K , Samson S E (1988) Effect of superoxide radical on Ca pumps of coronary artery Amer J Physiol **255,** C297—C303

Grover A K Samson S E (1989) Protection of Ca^{2+}-pump of coronary artery against inactivation by superoxide radical Amer J Physiol **256,** C666—C673

Grover A K , Jones T R , Daniel E E (1980) Effect of vanadate on rat myometrium plasma membrane enzyme activities Can J Physiol Pharmacol **58,** 1247—1250

Grover A K , Samson S E , Lee R M K W (1987) Effects of adenosine, nitroglycerin and carbachol on the large and small coronary arteries of the pig IRCS Med **15,** 577

Grover A K , Fomin V P , Samson S E (1992) Peroxide inactivates calcium pumps in pig coronary artery Amer J Physiol **263,** H537—H543

Kaneko M , Lee S -L , Wolf C M , Dhalla N S (1989) Reduction of calcium channel antagonist binding sites by oxygen free radicals in rat heart J Mol Cell Cardiol 21, 935—943

Kaneko M , Chapman D C , Ganguly P K , Beamish R E Dhalla N S (1991) Modification of cardiac adrenergic receptors by oxygen free radicals Amer J Physiol 260, H821—H826

Kim M -S , Akera T (1987) O_2 radicals cause of ischemia-reperfusion injury to cardiac Na^+-K^+-ATPase Amer J Physiol 252 H252—H257

Kirshenbaum L A Singal P K (1992) Changes in antioxidant enzymes in isolated cardiac myocytes subjected to hypoxia-reoxygenation Lab Invest **67,** 796—803

Kramer J H Mak I T Weglicki W B (1984) Differential sensitivity of canine cardiac sarcolemmal and microsomal enzymes to inhibition by free radical- induced lipid peroxidation Circ Res **55,** 120 —124

Kukreja R. C., Weaver A. B., Hess M. L. (1990): Sarcolemmal Na^+-K^+-ATPase: inactivation by neutrophil-derived free radicals and oxidants. Amer. J. Physiol. **259,** H1330—H1336

Rajagopalan K. V. (1985): Purification of bovine milk xanthine oxidase. In: Handbook of Methods for Oxygen Radical Research (Ed. R.A. Greenwald), pp. 21–23, CRC Press, Boca Raton, Florida

Rangachari P. K., Grover A. K., Daniel E. E. (1983): Thallium uptake by canine iliac arteries. Hypertension **5,** 436—441

Roberts M. J., Young I. S., Trouton T. G., Trimble E. R., Khan M. M., Webb S. W., Wilson C. M., Patterson G. C., Adgey A. A. (1990): Transient release of lipid peroxides after coronary artery balloon angioplasty. Lancet **336,** 143–145

Samaha F. F., Heineman F. W., Ince C. (1992): ATP-sensitive potassium channel is essential to maintain coronary vascular tone in vivo. Amer. J. Physiol. **262,** C1220—C1227

Todoki K., Okabe E., Kiyose T., Sekishita T., Ito H. (1992): Oxygen free radical-mediated selective endothelial dysfunction in isolated coronary artery. Amer. J. Physiol. **262,** H806—H812

Vlessis A. A., Muller P., Bartos D., Trunkey D. (1991): Mechanism of peroxide-induced cellular injury in cultured adult cardiac myocytes. FASEB J. **5,** 2600—2605

Xie Z., Wang Y., Askari A., Huang W. -H., Klaunig J. E., Askari A. (1990): Studies on the specificity of the effects of oxygen metabolites on cardiac sodium pump. Mol. Cell Cardiol. **22,** 911—920

Zweier J. (1988): Measurement of superoxide-derived free radicals in the reperfused heart. J. Biol. Chem. **263,** 1353—1357

Final version accepted May 18, 1994