Free Rad. Res. Comms., Vol. 1, No. 2, pp. 123–128
8755-0199/85/0102-0123 $25.00/0

Printed in Great Britain

EFFECT OF ISCHEMIA ON HEART SUBMITOCHONDRIAL SUPEROXIDE PRODUCTION

C. GUARNIERI†, C. MUSCARI, C. VENTURA and I. MAVELLI††

Istituto di Chimica Biologica dell'Università di Bologna and ††Istituto di Biochimica Applicata dell'Università di Roma and Centro di Biologia Molecolare del C.N.R., Romá Italy

NADH-dependent formation of superoxide anions ($O_2^{\cdot-}$) by rabbit cardiac submitochondrial particles (SMP) was stimulated after exposure of the isolated heart to 90 min of ischemic perfusion. This effect was more evident in the rotenone-inhibited region of the respiratory electron chain in comparison to the antimycin-inhibited region. The kinetic study of the NADH-dependent reaction showed that at the level of the rotenone-inhibited region, ischemia reduced K_m value for NADH, differently from the antimycin-inhibited region where the kinetic constants remain unchanged. No significant changes of the V_{max} values were observed in both SMP-producing $O_2^{\cdot-}$ sites.

The ischemic perfusions also produced a reduction of mitochondrial function, particularly evident when glutamate as substrate was studied.

Key words: superoxide, mitochondria, ischemia, heart

Abbreviations used: $O_2^{\cdot-}$, superoxide anion; SMP, submitochondrial particles.

INTRODUCTION

Although ischemia is a condition characterized by low intracellular oxygen tensions, recent studies indicate that it promotes in several tissues, including intestine[1] and heart muscle[2], cellular injury mediated by reactive oxygen species such as the superoxide anion ($O_2^{\cdot-}$).

In the ischemic myocardium the major source of $O_2^{\cdot-}$ appears to be the enzyme xanthine oxidase in consequence of its conversion to the superoxide-producing oxidase form[3]. This conclusion is confirmed by the fact that pretreatment of the animals with allopurinol effectively reduced the ischemia-induced tissue changes[4]. In the heart muscle the mitochondrial electron transferring system can also function as a generator of $O_2^{\cdot-}$ radicals[5]. This production becomes particularly evident when the respiratory chain carriers located on the inner mitochondrial membranes are highly reduced[6]. Since ischemia favours the accumulation of cofactors or substrates which can effectively maintain the mitochondrial electron components in a reduced state, it is likely that the ischemic mitochondria may enhance $O_2^{\cdot-}$ production, especially if O_2

†Address to whom correspondence should be sent: Prof. C. Guarnieri, Istituto di Chimica Biologica, Via Irnerio, 48, 40126, Bologna, Italy.

is present in concentrations that limit its reduction to H_2O by the cytochrome oxidase (1 to 3 mmHg)[7]. In cardiac mitochondria, NADH dehydrogenase and the ubiquinone-cytochrome b region located on the inner membrane are the major source of $O_2^{\cdot}$ radicals[6].

The present study was designed to determine the role of these two mitochondrial sites on the formation of $O_2^{\cdot}$ when the cardiac muscle is exposed to ischemic conditions.

MATERIALS AND METHODS

Male New Zealand rabbits were used throughout this study. The animals were sacrificed and their hearts used for *in vitro* perfusion with Krebs-Henseleit bicarbonate buffer gassed with 95% O_2 - 5% CO_2 and containing 11 mM glucose as previously described[8]. Ischemia was induced by reducing the coronary flow from 20 ml/min (aerobic control perfusion) to 0.5 ml/min for 90 min. At the end of the perfusion the hearts were homogenized in 180 mM KCl, 10 mM EDTA and 0.5% bovine serum albumin, pH 7.2 using an Ultra-Turrax homogenizer and the mitochondria were prepared as described by Williams and Barrie[9]. Mitochondrial respiratory control index (RCI), state 3 oxygen consumption (QO_2) and the ADP/O ratio were calculated from the O_2-partial pressure measured by a Clark electrode fitted in a closed water jacketed at 25°C (Gilson Instruments, France). The assay medium consisted of 3 mM substrate (glutamate or succinate), 250 mM sucrose, 0.5 mM EDTA, 3 mM KH_2PO_4 at pH 7.4. ADP (250 μM) was added to the incubation mixture to initiate state 3-mitochondrial respiration. Submitochondrial particles (SMP) were prepared by sonicating the mitochondria (10 mg/ml) in EDTA 2 mM, pH 8.5, at 40 W four times for 15 s with a 15 s interval at 4°C in a Labsonic Sonifier cell disruptor[10].

The sonicated mitochondrial suspension was centrifuged at 10,000 g × 10 min and the resulting supernatant recentrifuged at 105,000 g × 30 min. The SMP pellet was washed twice with 250 mM sucrose/10 mM Tris-HCl, pH 7.4, by centrifugation at 105,000 g × 30 min. The washed particles were essentially free from superoxide dismutase, as shown by activity measurements according to Guarnieri *et al.*[11].

Production of $O_2^{\cdot}$ by SMP was measured by the superoxide dismutase-sensitive oxidation of adrenaline to adrenochrome[12]. The assay system included 250 mM sucrose/50 mM HEPES, pH 7.5, 1 mM adrenaline and 200-300 μg SMP protein.

TABLE I
Mitochondrial respiratory and electron transport enzymatic changes caused by 90 min of ischemic perfusion on isolated rabbit heart

	RCI		QO_2		ADP/O	
	Glutamate	Succinate	Glutamate	Succinate	Glutamate	Succinate
Control	15.2 ± 2.8	4.4 ± 0.5	166.5 ± 4.5	175.5 ± 7.5	2.78 ± 0.31	1.65 ± 0.06
Ischemia	5.7 ± 0.8	2.6 ± 0.2	134.0 ± 8.0	170.1 ± 12.0	2.20 ± 0.38	1.58 ± 0.2
P	< 0.001	< 0.02	< 0.001	< 0.01	< 0.05	N.S.

RCI, respiratory control index calculated as ratio of oxygen consumed in the presence of ADP to that taken up after phosphorylation of ADP; QO_2, n atoms oxygen uptake in presence of ADP/mg mitochondrial protein·min; ADP/O, n mol ATP produced/n atom oxygen consumed.

The results are expressed as mean ± SE of four separate experiments. P indicates significant differences.

TABLE II
Production of $O_2^{\cdot}$ by SMP from rabbit ischemic heart

	Rotenone-inhibited region[a]	Antimycin-inhibited region[b]
Control	4.01 ± 0.1[c]	2.20 ± 0.1
Ischemia	4.92 ± 0.2	2.85 ± 0.2
P	< 0.02	< 0.05

The production of $O_2^{\cdot}$ was evaluated as described in Materials and Methods, by incubating the SMP with (a) 1.5 μM rotenone or (b) 2 μM antimycin. The data in (b) experiments were calculated by subtracting the $O_2^{\cdot}$ rate determined in the presence of rotenone from those obtained with antimycin.

(c) The values expressed as n mol $O_2^{\cdot}$/min·mg prot represent the means ± S.E. of four separate experiments; P indicates significant differences.

Absorbance changes were monitored at 25°C in a Perkin Elmer double beam spectrophotometer at 480 nm by adding NADH 0.125 mM ($E = 4.02\ mM^{-1}\ cm^{-1}$). These measures were carried out during the initial rates (1 min) for considering the adrenochrome formation representative of true rates of $O_2^{\cdot}$ generation.

Protein determinations were made by the method of Bradford[13] using bovine serum albumin as standard.

RESULTS

Ischemic perfusion of the isolated rabbit heart resulted in a significant depression in all the parameters of mitochondrial function (Table I). This effect was particularly evident when glutamate, a NAD-linked substrate was studied. In fact in this case the RCI, QO_2 and ADP/O ratio values were significantly depressed ($p < 0.001$; < 0.001; < 0.05, respectively). On the other hand, by using succinate as substrate, the RCI and QO_2 values of the ischemic mitochondria were reduced with respect to the control ($p < 0.02$; < 0.01, respectively), whereas the ADP/O value remained unchanged.

Table II shows that the production of $O_2^{\cdot}$ by the SMP supplemented with NADH is almost double in the rotenone-inhibited region with respect to the value determined in the site located in the antimycin sensitive region. Due to the effect of the ischemic perfusion, the $O_2^{\cdot}$ production was more elevated in both sites, with a more pronounced stimulating effect at the level of the rotenone-inhibited region ($p < 0.02$).

Figure 1 represents the kinetic behaviours of the NADH-dependent $O_2^{\cdot}$ production by the rotenone-inhibited region of SMP prepared from control and ischemic hearts. Ischemic SMP showed a decreased value of K_m for NADH in comparison to the control, while V_{max} values were rather similar in both cases.

Figure 2 shows.that no significant changes were observed in the kinetic plots concerning the NADH stimulated-$O_2^{\cdot}$ formation by the antimycin-inhibited region of control or ischemic SMP.

DISCUSSION

This study evidentiates that SMP prepared from ischemic cardiac muscle produce more $O_2^{\cdot}$ than control SMP. This event is particularly significant at the level of the rotenone-inhibited mitochondrial region of the respiratory chain, and from the

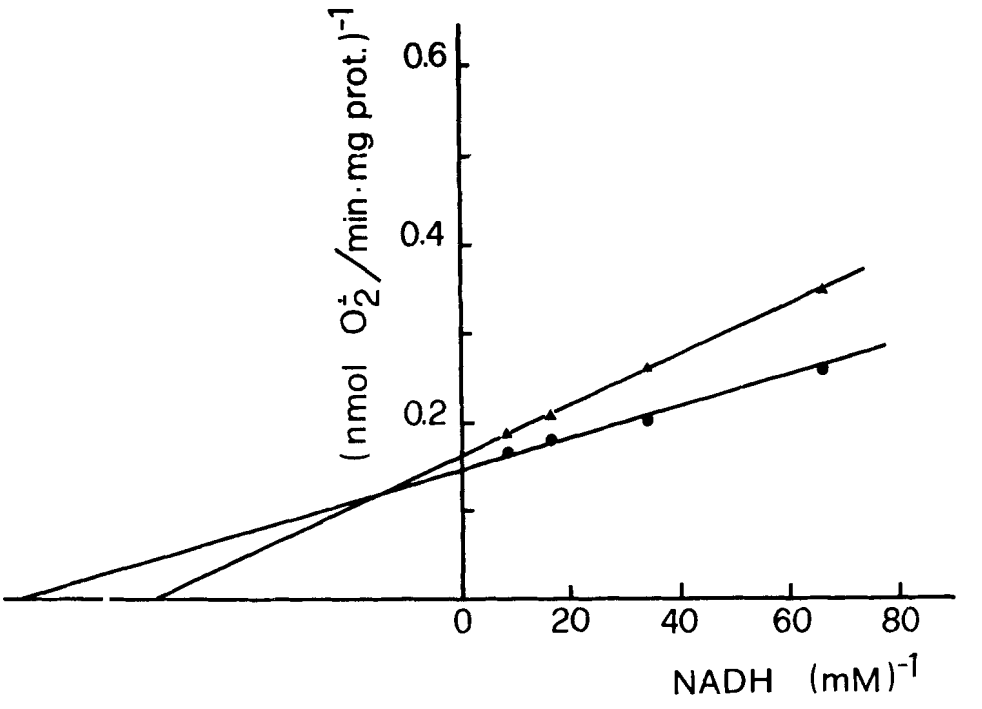

FIGURE 1 Effect of ischemia on the NADH-stimulated $O_2^{\cdot}$ formation by rotenone-inhibited region of heart SMP. Production of $O_2^{\cdot}$ was measured as described in Materials and Methods section adding 1.5 μM rotenone in the incubation mixture.

(-▲-) Control; (-●-) Ischemia.

kinetic study it appears that this stimulation could be the consequence of an augmented affinity shown by the ischemic SMP for the cofactor NADH. Less marked was the stimulatory effect measured at the level of the antimycin-inhibited mitochondrial region, where the kinetic study reveals no significant differences of the kinetic parameters between control and ischemic SMP. The augmented affinity for NADH is likely to be able to induce an increased $O_2^{\cdot}$ production by the NADH dehydrogenase. In fact, since the ischemic mitochondria showed a decreased ability to oxidize substrates, particularly NAD-linked (Table I), it is probable that this effect favours the maintenance of a highly reduced state of the flavoprotein located in the rotenone-inhibited site, involved in the $O_2^{\cdot}$ formation by its spontaneous autoxidation[6]. According to the mechanism proposed by Bielski and Chan[14], it is highly possible that NADH bound to dehydrogenase, generates more $O_2^{\cdot}$ due to the effect of the ischemia-induced mitochondrial damages.

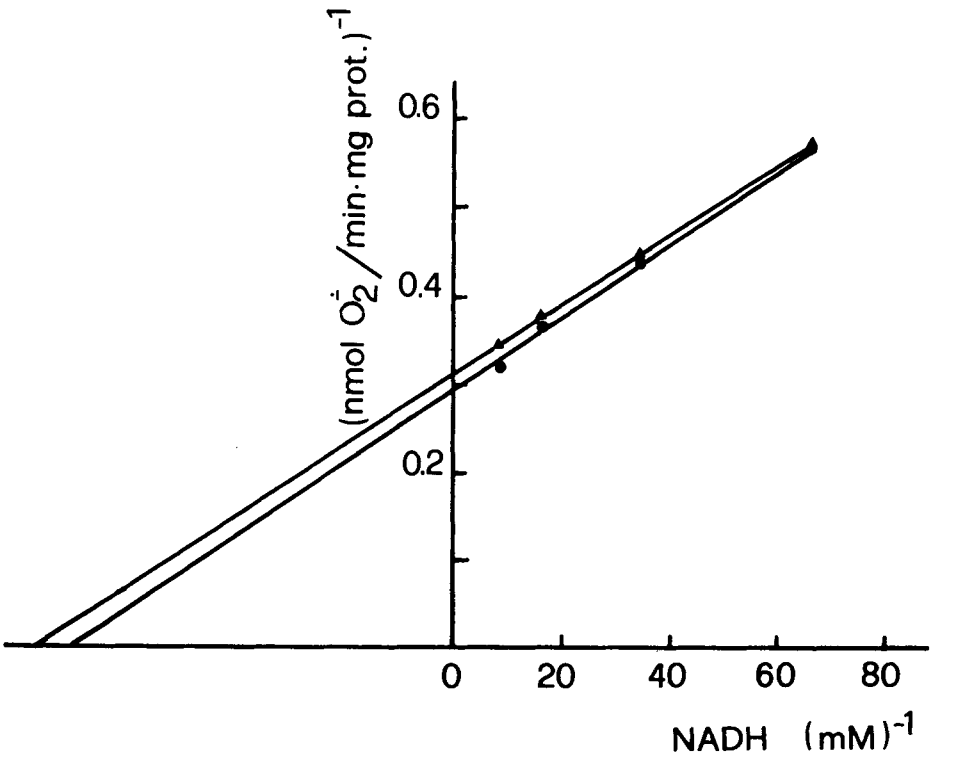

FIGURE 2 Effect of ischemia on the NADH-stimulated $O_2^{\cdot}$ formation by antimycin-inhibited region of heart SMP. Production of $O_2^{\cdot}$ was measured as described in Materials and Methods section adding 2 μM antimycin in the incubation mixture. Calculations were made according to legend in Table II.

(-▲-) Control; (-●-) Ischemia.

Also in the heart SMP after incubation with adriamycin, the NADH dehydrogenase produced an increased amount of $O_2^{\cdot}$[15], thus indicating that this mitochondrial electron complex is particularly suitable and versatile to generate $O_2^{\cdot}$.

Values of $O_2^{\cdot}$ formation higher than normal were also obtained with heart SMP prepared from old rats[16], or from rabbits which had undergone prolonged cardiac hypertrophy[17], or from rats exposed to hyperbaric oxygenation[18]. Whole mitochondria extracted from infarcted heart muscle also produced more $O_2^{\cdot}$ as revealed by electron spin resonance by Suzuki[19].

Since $O_2^{\cdot}$ radicals are toxic for mitochondrial structure[16,20] and function[21], it is possible that an increased production of them during ischemic stress, concomitantly to a loss of efficiency of the enzymatic and metabolic $O_2^{\cdot}$-scavenger mechanisms[22,23,24], may be responsible for the formation of the ischemia-induced mitochondrial damages.

Such a hypothesis is confirmed by experiments carried out in the presence of $O_2^{\cdot}$ scavengers during ischemic perfusion of the heart muscle, which showed an improved mitochondrial function[25].

Acknowledgements

The authors would like to thank Professor C.M. Caldarera and Professor G. Rotilio for their advice and encouragement, and the Ministero della Pubblica Istruzione (Rome) for its financial support.

References

1. D.A. Parks and D.N. Granger, in Oxy Radicals and their Scavenger Systems, ed. R.A. Greenwald and G. Cohen (Elsevier Science Publishing, New York, 1983) vol. 2, p. 135.
2. P.S. Rao, M.V. Cohen and H.S. Mueller, *J. Mol. Cell. Cardiol.*, **15**, 713, (1983).
3. R.S. Ray and J.M. McCord, *Fed. Proc.*, **41**, 767, (1982).
4. R.A. De Wall, K.A. Vasko, E.L. Stanley and P. Kezdi, *Am. Heart J.*, **82**, 362, (1971).
5. G. Loschen, A. Azzi, C. Richter and L. Flohé, *FEBS Lett.*, **42**, 68, (1974).
6. J.F. Turrens, B.A. Freeman and J.D., Crapo, *Arch. Biochem. Biophys.*, **217**, 411, (1982).
7. F.F. Jobsis and J.D. La Manna, in Extrapulmonary Manifestation of Respiratory Disease, ed. E.G. Robin (Dekker, New York, 1978), p. 63.
8. C. Guarnieri, F. Flamigni and C.M. Caldarera, *J. Mol. Cell. Cardiol.*, **12**, 797, (1980).
9. A.J. Williams and S.E. Barrie, *Biochem. Biophys. Res. Commun.*, **84**, 89, (1978).
10. H. Nohl and D. Hegner, *Eur. J. Biochem.*, **82**, 563, (1978).
11. C. Guarnieri, F. Flamigni, C. Ventura and C. Rossoni Caldarera, *Biochem. Pharmacol.*, **30**, 2174, (1981).
12. J.F. Turrens and A. Boveris, *Biochem. J.*, **191**, 421, (1980).
13. M.M. Bradford, *Anal. Biochem.*, **72**, 246, (1976).
14. B.H.J. Bielski and P.C. Chan, in Superoxide and Superoxide Dismutase, eds. A.M. Michelson, J.M. McCord and I. Fridovich (Academic Press, London, 1977), p. 409.
15. K.J.A. Davies, J.H. Doroshow and P. Hochstein, *FEBS Lett.*, **153**, 227, (1983).
16. H. Nohl, V. Breuninger and D. Hegner, *Eur. J. Biochem.*, **90**, 385, (1978).
17. C. Guarnieri, C. Muscari and C.M. Caldarera, in Advances in Myocardiology, eds. P. Harris and P.A. Poole-Wilson (Plenum Publishing Corporation, New York, 1985), p. 191.
18. H. Nohl, D. Hegner and K.H. Summer, *Biochem. Pharmacol.*, **30**, 1753, (1981).
19. Y. Suzuki, *Jap. Circulation J.*, **39**, 683, (1975).
20. F. Flamigni, C. Guarnieri, R. Toni and C.M. Caldarera, *Int. J. Vit. Nut. Res.*, **52**, 402, (1982).
21. C. Guarnieri, C. Muscari, F. Flamigni and C.M. Caldarera, *J. Mol. Cell. Cardiol.*, **15**, 859, (1983).
22. P.S. Rao and H.S. Mueller, *Clin. Chem.*, **27**, 1027, (1981).
23. H.M. Rinette, L.B.M. Tijburg, L. Sterrenberg, A. Bast, J.M. Koomen and J. Moordhoek, *Life Sci.*, **35**, 1281, (1984).
24. R. Ferrari, C. Ceconi, S. Curello, C. Guarnieri, C.M. Caldarera and O. Visioli, *J. Mol. Cell. Cardiol.* in press.
25. C. Guarnieri, R. Ferrari, O. Visioli, C.M. Caldarera and W.G. Nayler, *J. Mol. Cell. Cardiol.*, **10**, 893, (1978).

Accepted by Professor G. Rotilio