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EFFECT OF REDUCED OXYGEN INTERMEDIATES ON SARCOLEMMAL MUSCARINIC RECEPTORS FROM CANINE HEART

Rakesh C. Arora and Michael L. Hess*

Department of Medicine, Division of Cardiology, Medical College of Virginia
Richmond, Virginia 23298

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SUMMARY. The effect of oxygen free radicals generated by xanthine-xanthine oxidase system and hydrogen peroxide were investigated on cardiac muscarinic cholinergic receptors. We have used highly enriched sarcolemmal preparations isolated from canine myocardium. Exposure of the sarcolemma to oxygen free radicals by xanthine-xanthine oxidase system resulted in a significant ($P < 0.05$) decrease of B_{max} of (^{3}H)-QNB (4.66 $\pm$ 0.51 to 2.68 $\pm$ 0.22 pmoles/mg protein). Addition of superoxide dismutase (SOD) and catalase (10µg/ml) resulted in a significant reversal of B_{max} value to 3.72 $\pm$ 0.39 pmoles per mg protein ($p < 0.05$). However, the affinity constants of dissociation (K_D) were not altered appreciably with the exposure to oxygen free radicals with or without scavengers. Hydrogen peroxide significantly depressed ^{3}H-QNB binding to the receptors in a dose-dependent manner in a concentration range between 4.41 mM - 441 mM. This depression was completely inhibited by 10 µg/ml catalase. The study demonstrates that the oxygen free radical species are capable of disrupting (^{3}H)-QNB binding to the cardiac muscarinic receptors.

Several recent studies utilizing the radiolabelled muscarinic antagonist (^{3}H)-quinuclidlinylbenzilate (QNB) have provided direct evidence of the presence of muscarinic cholinergic receptors in ventricular myocardium (1,2,3,4). The role of these muscarinic receptors in the modulation of biochemical and electrophysiological events at the cellular level is a topic of considerable interest.

Partially reduced and thereby activated oxygen species are increasingly implicated as potential mediators of myocardial injury (5). Oxygen free radicals have been shown to disrupt calcium transport by both cardiac and skeletal muscle sarcoplasmic reticulum (6,7). Using a xanthine-xanthine oxidase system, these authors proposed that the species of free radical generated are pH dependent and that both superoxide and hydroxyl radicals depress calcium transport in sarcoplasmic reticulum. In the present communication, for the first

*To whom correspondence should be addressed.

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time, we report the effects of oxygen free radicals generated by xanthine-xanthine oxidase system and exogenous hydrogen peroxide on ^{3}H-QNB binding to cardiac muscarinic cholinergic receptors of isolated sarcolemma from canine heart.

MATERIALS AND METHODS

Chemicals: (^{3}H)-QNB (51.6 Ci/mmol) was purchased from New England Nuclear. Atropine, xanthine, xanthine oxidase (0.56 units/mg protein), aprotinin agarose, superoxide dismutase (SOD), catalase, and hydrogen peroxide were obtained from Sigma Chemical Company.

Preparation of Sarcolemmal Vesicles: Sarcolemmal vesicles were isolated according to the method of Alstyne et al. (8). Dogs were anesthetized with pentobarbital (30 mg/kg body weight) intravenously, the heart rapidly removed and immersed in ice-cold saline. All procedures were performed at 4°C. The heart was stripped of epicardial and endocardial tissue, finely minced, suspended in 4-5 vols 10 mM $NaHCO_3$ and 5 mM NaN_3, pH 7.0 (Medium I) and homogenized with three low speed 15 sec pulses (separated by 30 sec intervals) in a Sorvall omnimixer. The homogenate was centrifuged at 8700 x g_{max} for 20 min. The supernatants were discarded and the pellets resuspended in the original volume of Medium I and homogenized with one pass of a motor driven Teflon pestle and centrifuged at 8700 x g_{max} for 20 min. The supernatants were discarded and the pellets resuspended in 6 vols of 10 mM Tris-HCl, pH 7.4 (Medium II) and subjected to 4 passes of a motor driven pestle. The homogenates were next centrifuged at 8700 x g_{max} for 20 min. All pellets were collected and resuspended to a total volume of 20 ml and homogenized by 5 passes of a hand driven teflon pestle in a homogenizing vessel. This suspension was layered over 15 ml of a 24% (w/v) sucrose solution containing 10 mM Tris-HCl, pH 7.4 and centrifuged at 73,400 x g_{max} for 30 min. The white membrane material at the buffer/sucrose interface was collected by aspiration with a pasteur pipette, diluted with 50 ml of Medium II and centrifuged at 73,400 x g_{max} for 20 min.

The pellet was resuspended in medium II to a final protein concentration of approximately 2 mg/ml as determined by the method of Lowry et al. (9). The preparation was stored at 4°C until the determination of (Na^+, K^+)-ATPase activity (usually 16 hours). The sarcolemma could be stored frozen at -20°C for several days without any appreciable effect on muscarinic receptor binding.

Assay of (Na^+, K^+)-ATPase activities: Ouabain sensitive (Na^+, K^+)-ATPase activities were measured by the method of Jones et al. (10). The reaction mixture, in a total volume of 1 ml, consisted of 100 mM NaCl, 10 mM KCl, 3 mM $MgCl_2$, 5 mM NaN_3, 1 mM EGTA, 50 mM Histidine, 3 mM Na-ATP and approximately 50 µg of sarcolemmal protein. To reveal total (Na^+, K^+)-ATPase activity, membrane vesicle preparations were preincubated with an optimal concentration of Lubrol PX (6 mg/mg protein). (Na^+, K^+)-ATPase activity was taken as that activity inhibitable by 1 mM ouabain. Membranes were assayed at 37°C for (Na^+, K^+)-ATPase activity and inorganic phosphorous released from ATP was measured colorimetrically (11).

Muscarinic Receptor Binding Assay: Assay of membrane-bound muscarinic receptors was performed by the previously described method of Fields et al. (1). Membrane vesicles (approximately 25 - 70 µg protein) were incubated in duplicate in 5 ml of medium containing 50 mM Tris-HCl (pH 7.4 at 25°C) and 20-1000 pM concentrations of (^{3}H)-QNB in presence or absence of 1 µM atropine. Incubations were conducted at 37°C for 60 min in a shaking water bath. Incubations were terminated by rapid filtration through Whatman GF/C filters. The filters were dried at 60°C overnight and suspended in 5 ml of Budget Solve counting cocktail. The samples were counted in a Packard counter using DPM program.

Generation of Oxygen Free Radicals: Oxygen free radicals were generated by xanthine oxidase (0.03 units/ml) acting on xanthine (0.15 mM) as a substrate. It has been shown that xanthine oxidase generates superoxide anion radical ($\cdot O_2^-$), which upon dismutation by SOD produces H_2O_2 (12) or free hydroxyl radical ($\cdot OH$) may be generated by interaction of ($\cdot O_2^-$) and H_2O_2 (13,14), which is a powerful oxidizing agent.

Xanthine oxidase was freed of protease activity by treating the enzyme with aprotinin-agarose before using in muscarinic receptor binding assay. SOD and catalase (10µg/ml) were used to scavenge $\cdot O_2^-$ and H_2O_2, respectively.

Hydrogen peroxide (4.41 - 441 mM) in absence and presence of catalase (10 µg/ml) were included in the receptor binding assay system.

RESULTS

Enrichment of Sarcolemmal Vesicles: The average yield of sarcolemmal vesicles by this method was 4.68 ± 0.54 mg/100 g whole heart. The patent (Na^+, K^+)-ATPase activities in the final membrane was quite high (22.32 ± 0.66 µmol Pi/mg protein · hour, n=9). The activity in this fraction after treatment with Lubrol-PX was nearly three times higher than the patent activity (63.09 ± 0.66 µmol Pi/mg protein · hour, n=5). which was somewhat lower than the values 77-79 µmoles/mg per hour reported by Jones et al. (15) after treatment of a sarcolemma-enriched preparation from heart with SDS.

(^{3}H)-QNB Binding to Cardiac Muscarinic Receptors: A typical experiment showing the binding of (^{3}H) QNB to the enriched sarcolemma is shown in Fig. 1. The saturation isotherm resembled a rectangular hyperbola and the Scatchard analysis of the data shows a single population of saturable high affinity muscarinic cholinergic receptors.

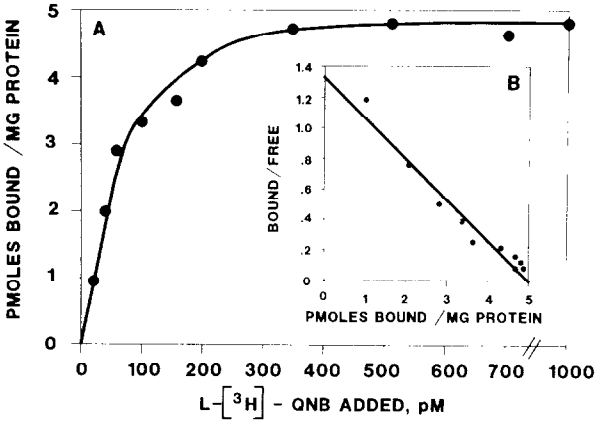

FIG. 1. Representative saturation isotherm of (^{3}H)-QNB binding to sarcolemmal vesicles (A). The specific binding was determined as the difference between total and non-specific bindings in parallel assays in the absence or presence of 1µM atropine. The inset (B) shows a Scatchard plot of the binding data.

TABLE 1

(^{3}H)-QNB binding parameters for cardiac sarcolemma exposed to oxygen free radicals in absence and presence of SOD and catalase

	Bmax pmoles/mg protein	K_D pM
Control (with xanthine)	4.66 ± .51	25.70 ± 9.51
Xanthine + Xanthine Oxidase	2.68 ± .22	28.70 ± 7.80
Xanthine + Xanthine Oxidase + SOD + Catalase	3.72 ± .39	30.00 ± 3.47

The values are mean ± SEM of 4 experiments.

Effect of Oxygen Free Radicals on (^{3}H-QNB Binding to Sarcolemmal Vesicles: The exposure of membrane fractions to exogenous oxygen free radicals by xanthine-xanthine oxidase resulted in the significant depression ($p < 0.05$) of Bmax of (^{3}H)-QNB from 4.66 ± 0.51 pmoles/mg protein to 2.68 ± 0.22 p moles/mg protein as shown in Table 1 and Fig. 2. The addition of SOD and catalase in the parallel experiments resulted in an increase of Bmax value to 3.72 ± 0.39 p moles/mg protein ($p < 0.05$). The decrease in Bmax following exposure to free oxygen radicals and subsequent partial reversal in Bmax by scavengers was not

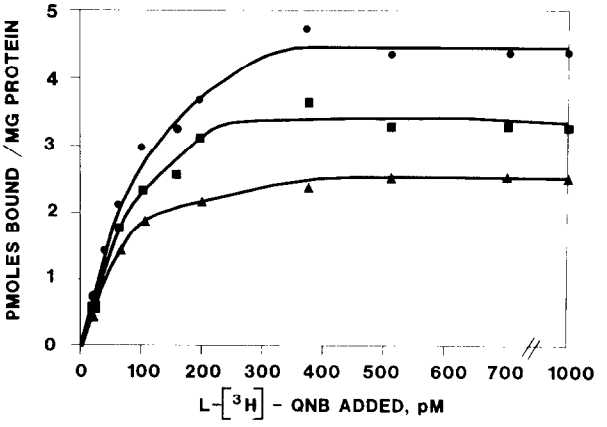

FIG. 2. Effect of oxygen free radicals on cardiac muscarinic receptors. Sarcolemma was incubated with 20 - 1000 pM (^{3}H)-QNB ± μM atropine in presence of xanthine (●) xanthine + oxidase (▲) and xanthine + xanthine oxidase + SOD + catalase (■) at 37°C for 60 minutes. Membrane bound receptors were separated by Millipore filtration technique.

associated with the similar concomitant significant changes in the K_D values (Table 1).

Effect of Hydrogen Peroxide on (^3H)-QNB Binding: The preceeding data appear to incriminate either hydrogen peroxide or the superoxide anion ($\cdot O_2^-$) as the disruptive species of oxygen free radicals that interact with sarcolemma. To test this possibility, a hydrogen peroxide concentration response study with and without catalase (10 µg/ml) was performed. Table 2 presents results of this study. Hydrogen peroxide significantly depressed (^3H) QNB-binding to muscarinic receptors in a dose response fashion in concentration range from 4.41 - 441 mM. This response was completely inhibited by catalase. Similar effects of hydrogen peroxide have been seen on (Na^+, K^+)-ATPase activity of the sarcolemma (data not shown).

DISCUSSION

The (Na^+, K^+)-ATPase, a sarcolemma marker which plays a major role in myocardial excitation-contraction coupling was highly enriched in the membrane fraction and its patent as well as total activities were comparable to those

TABLE 2

Effect of hydrogen peroxide on (^3H)- QNB binding activity to isolated cardiac sarcolemma

	(^3H) QNB Binding Activity (pmoles/mg protein)
Control	2.92 ± 0.16
441 mM H_2O_2	0.45 ± 0.12
441 mM H_2O_2 + Catalase (10 µg/ml)	2.69 ± 0.09
44.1 mM H_2O_2	1.80 ± 0.08
44.1 mM H_2O_2 + Catalase (10 µg/ml)	2.67 ± 0.14
4.41 mM H_2O_2	2.12 ± 0.25
4.41 mM H_2O_2 + Catalase (10 µg/ml)	2.80 ± 0.12

Sarcolemmal vesicles (25-50 µg) were incubated with 600 pM (^3H)-QNB ± 1 µM atropine in presence of 4.41 - 441 mM H_2O_2 ± 10 µg/ml catalase at 37°C for 60 minutes in a shaking bath. Bound activity was obtained as described in the Materials and Methods. The results are mean ± SEM of 4 experiments.

reported by others (8,15). The binding isotherms of (^{3}H) QNB and Scatchard analysis (Fig. 1) of the membranes from the top layer were consistent with the presence of one binding component with a dissociation constant (K_D) of 25.7 $\pm$ 9.7 pM and receptor density (Bmax = 4.66 $\pm$ .51 p moles/mg protein) similar to the values reported by Manalan et al. (4) in the enriched fractions of canine sarcolemmal vesicles.

In the heart, acetylcholine mediates chronotropic and inotropic effects in both the atria and ventricles via muscarinic cholinergic receptors, which are located on the sarcolemma and on the terminals of post-ganglionic cholinergic and possibly adrenergic neurons. This distribution of acetylcholine sensitive sites provide the milieu for complex functional interactions between the parasympathetic nervous system and myocardial function. Oxygen free radicals have been implicated as mediators of cell injury in several pathological conditions such as oxygen toxicity (16), ischemia of the central nervous system (17), radiation injury (18), cerebral vascular damage from hypertension (19). Previous work from our laboratory has implicated oxygen free radicals in the pathogenesis of ischemia/reperfusion injury and leukocytic infiltration of the myocardium (7,21,22). However, this work was based on oxygen free radical disruption of sarcoplasmic reticulum function. The sarcoplasmic reticulum of cardiac muscle is intracellular and particularly with leukocytic infiltration of the myocardium the radical would first be generated in the extracellular space. This study clearly demonstrates that oxygen free radicals are capable of disrupting the sarcolemma of cardiac muscle (as shown by disruption of markers) which would then permit access of the intracellular space to the extracellular environment.

It is thought that sarcolemmal membrane enzymes and receptors are also susceptible to damage by the oxygen free radicals. Our results show significant decrease in receptor densities as indicated by depression in Bmax of (^{3}H) QNB binding to isolated sarcolemma, by using xanthine-xanthine oxidase system. However, the observation that the inhibition of (^{3}H)-QNB binding is partially reversed by SOD and catalase may be explained due to the additional generation

of hydroxyl radical since our data clearly show that the toxic effects of exogenously added hydrogen peroxide to the binding assay system are completely inhibited by catalase (Table 3).

It has been reported that trypsin causes a decrease in (Na^+, K^+)-ATPase and calcium binding activities in the sarcolemma (20). The xanthine-oxidase enzyme prepared from buttermilk (Sigma) is contaminated with approximately 5% protease activity. We, therefore, eliminated this possibility in our study by treating xanthine oxidase with aprotinin-agarose in order to inhibit protease activity present in the enzyme. Further, the dose-dependent inhibition of (^3H)-QNB binding to muscarinic receptor binding by exogenously added hydrogen peroxide, clearly shows the role of this species in disrupting (^3H)-QNB binding to the receptor. Similar effects of hydrogen peroxide have been seen in the (Na^+, K^+)-ATPase activity (data not shown).

Previous work from this laboratory has suggested that xanthine-xanthine oxidase interaction and neutrophil generation of reduced oxygen intermediates are able to disrupt sarcoplasmic reticular calcium transport, and that the system was inhibitable by SOD and catalase (21,22). The present data demonstrate that cardiac muscle sarcolemma may also be an important target organelle for the oxygen free radical damage.

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REFERENCES

1. Fields, J.Z., Roeske, W. R., Moykin, E., and Yamamura, H.I. (1978) J. Biol. Chem. 253, 3251-3258.
2. Wei, J.W., and Sulakhe, P.V. (1978) Eur. J. Pharmacol. 53, 235-238, 1978.
3. Mirro, M.J., Mamalan, A.S., Bailey, J.C., and Wantanabe, A.M. (1980) Circ. Res. 47, 855-865.
4. Manalan, A.S., Werth, D.K., Jones, L.R., and Wantanabe, A.M. (1983) Circ. Res. 52, 664-676.
5. Hammond, B., and Hess, M.L. (1985) J. Am. Coll. Cardiol. In Press.
6. Hess, M.L., Okabe, E., and Kontos, H.A. (1981) J. Mol. Cell. Cardiol. 13, 1375-1379.
7. Okabe, E., Hiyama, E., Oyama, M., Odajima, C., Ito, H., Cho, Y. (1982) 25, 138-148.

8. Alstyne, E.V., Burch, R.M., Knickelbein, R.G., Hungerford, R.T., Grower, E.J., Webb, J.G., Poe, S.L., and Lindenmayer, E. (1980) Biochim. Biophys. Acta 602, 131-143.
9. Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J. (1951) J. Biol. Chem. 193, 265-272.
10. Jones, L.R., Besch, H.R., Jr., and Wantanabe, A.M. (1977) J. Biol. Chem. 252, 3315-3323.
11. Penney, C.L. (1976) Anal. Bioch. 75, 201-210.
12. Fridovich, I. (1970) J. Biol. Chem. 245, 4053-4057.
13. Haber, F., and Weiss, J. (1934) J. Proc. Royal Soc. 147, 332-351.
14. McCord, J.M., and Fridovich, I. (1968) J. Biol. Chem. 243, 5753-5760.
15. Jones, L.R., Besch, H.R., Jr., Fleming, J.W., McConnaughey, M.M., and Watanabe, A.M. (1979) J. Biol. Chem. 254, 530-539.
16. Fridovich, I. (1978) Science 201, 875-880.
17. Demopoulous, H.B., Flamm, E.S., Seligman, M.L., Mitamura, J.A. and Rsnsohoff, J. (1979) In: Popp, A.J., Bourke, R.S., Nelson, L.R., and Kimelberg, H.K. Eds. Neural Trauma, New York: Raven Press, 63-78.
18. Misra, H.P. and Fridovich, I. (1972) The generation of superoxide radical during autooxidation of hemoglobin. J. Biol. Chem. 247, 6960-6962.
19. Kontos, H.A., Wei, E.P., Dietrich, W.D., Navari, R.M., Povlishock, J.T., Ghatak, N.R., Ellis, E.F., and Patterson, J.L., Jr. (1981) Am. J. Physiol. 240, H511-H527.
20. Dhalla, N.S., Anand-Srivastava, M.G., Tuana, B.S., and Khandelival, R.L. (1981) J. Mol. Cell. Cardiol. 13, 413-423.
21. Hess, M.L., Manson, N.H., Okabe, E. (1982) Can. J. Phys. and Pharmacol. 60, 1382-1389.
22. Rowe, G.T., Manson, N.H., Caplan, M., and Hess, M.L. (1983) Circ. Res. 53, 584-591.