Research Paper

Effect of reactive oxygen species on cholinergic receptor function

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ABSTRACT

Objective: To investigate the role of reactive oxygen species (ROS) on cholinergic receptor function.

Materials and Methods: Rectus abdominis and isolated heart preparations of frog (*Rana tigirina*) were used to assess nicotinic and muscarinic receptor activity, respectively. Thirty percent hydrogen peroxide (H_2O_2) solution and Fenton mixture (Fm, 13.9 mg, 50 µM of $FeSO_4$, 75 mg of sodium EDTA and 50 µL of 30% H_2O_2 were added to 10 ml of 0.1 M K_2HPO_4) were used to generate 1 mM H_2O_2 and hydroxyl free radicals. The responses were recorded with acetylcholine at different phases of exposure of tissues to ROS. Normal frog Ringer was used as a physiological solution. Responses of acetylcholine were also recorded in the presence of ROS before and after exposure of the tissue to an antioxidant (ascorbic acid).

Results: Free-radical-mediated receptor damage was dose (1–100 mM H_2O_2) and time (10–30 min) dependent when responses were taken with 30 µg and 30 ng of ACh for nicotinic and muscarinic receptors, respectively. There was no effect of ROS on prior exposure of tissue to ascorbic acid (antioxidant) at a concentration of 300 µg/ml. The antioxidant has not shown any beneficial effect on sulfhydryl groups of G-protein-coupled muscarinic receptors, which are more susceptible and sensitive to ROS than ion-channel nicotinic receptors where there is 96% protection with the antioxidant. Reactive oxygen species has shown different effects on receptor function.

Conclusion: Free radicals continuously cause considerable damage to the receptors. G-protein-coupled muscarinic receptors are more susceptible than ion-channel-linked nicotinic receptors. Antioxidants are shown to play a major role in protecting free-radical-mediated receptor damage.

KEY WORDS: Antioxidants; free radical; acetylcholine; ascorbic acid.

Introduction

Receptors are specialized to recognize and respond to individual signaling molecules with great selectivity. Proteins, glycoprotein, proteolipids, and associated proteinaceous matter constitute receptors.[1] Free radicals are chemical species possessing an unpaired electron that can be considered as a fragment of molecules which are generally very reactive. They are produced continuously in cells either as accidental by-product of metabolism or deliberately during phagocytosis. Reactive oxygen species (ROS) includes not only oxygen free radicals (OFR) but also nonradical oxygen derivatives that are involved in oxygen radical production. Hydrogen peroxide (H_2O_2) easily breaks down, particularly in the presence of transition metal ions to produce the most reactive OFR, the hydroxyl radicals.

Oxygen-derived free radicals and other reactive oxygen species have been shown to be important mediators of cellular and tissue injuries in a variety of diseases such as diabetes, heart failure, hypertension, and ischemia/reperfusion. Further, ROS are mediators of lipid peroxidation, protein and nucleic acid modification, which can result in altered cellular responses and cell death.[2] It has been recognised that free radicals are involved in the etiology of many diseases. Although insight into the role of free radicals in physiology and pathogenesis has been gained over the recent years, remarkably little knowledge exists on the effect of free radicals on receptor-mediated response.[3]

Imbalance between production of OFR and antioxidant defense can result in oxidative stress leading to metabolic impairment and cell death. Oxidative stress may be due to deficiency of antioxidants (such as glutathione, ascorbate or

α-tocopherol), antioxidant enzymes [superoxide dismutase (SOD), catalase, glutathione peroxidase] and/or from increased levels of OFR.[4] Several definable diseases arise from disorders in receptors or receptor–effector systems, such as *feminization syndrome, generalized endocrinopathy* and *pseudohypoparathyrodisim type 1a.*[5] Nicotinic receptors in myasthenia gravis,[6] LDL receptors in familial hypercholesterolemia, V_2 receptors in nephrogenic diabetes insipidus, ACTH receptors in cortical insufficiency, PTH receptors in pseudohypoparathyroidism and ß-adrenoceptors in hypertensive states are implicated in the free radical damage of receptors.[7]

The generation of ROS has been observed under various pathological conditions. In rat atria exposed to ROS, adrenoreceptor stimulation reduces the contractile force as a result of a protein kinase C-mediated Na^+/K^+-ATPase activation.[8] Activation of the M_1 muscarinic receptor subtype in rat pheochromocytoma cells stably expressing cloned M_1 muscarinic acetylcholine receptors was previously shown to induce morphological changes and growth arrest. However, signalling pathways, which led to these effects, were not identified.[9]

So far, the influence of ROS on the cardiac muscarinic receptors has not been studied in detail. The only studies so far performed indicate that the free radicals and H_2O_2 can alter the binding characteristics of the cardiac M_2 receptor, while the functional data are not yet available.[10] Reactive oxygen species act as second messengers in muscarinic-induced cellular signalling. Moreover, generation of ROS appears to be an early and critically intermediary event, which occurs immediately after stimulation of the muscarinic receptor and in turn affects the muscarinic-mediated cellular signalling.[9] Thus, receptors are prone to be damaged by the effects of free radicals. Even the pathogenesis of free-radical-mediated receptor dysfunction on cholinergic receptors is not well defined. In the present study, an attempt has been made to study these effects *in vitro*.

Materials and methods

Drugs and their source

Ferrous sulfate ($FeSO_4$), sodium EDTA, dipotassium hydrogen orthophosphate (K_2HPO_4), H_2O_2 and ascorbic acid (AA) were purchased from SD. Fine Chemicals, Mumbai, India. Acetylcholine was procured from LOBA Chemie Co., Mumbai, India. The chemicals required for the preparation of frog Ringer were purchased from SD. Fine Chemicals, Mumbai, India. All chemicals used were of analytical grade.

Preparation of solutions

Fenton mixture (*Fm*): The method of preparation of Fm is similar to that reported earlier.[11] About 13.9 mg ferrous sulfate 7 H_2O, 75 mg of sodium EDTA and 50 μl of 30% H_2O_2 were added to 10 ml 0.1 M dipotassium hydrogen orthophosphate solution and the reaction mixture was kept in a water bath at 40°C for 20 min with continuous stirring and the solution was used as a source of hydroxyl free radicals.

Frog Ringer solution: The physiological salt solution was prepared by adding NaCl 110 mM, KCl 1.9 mM, $CaCl_2$ 1.1 mM, $NaHCO_3$ 2.4 mM, and Glucose 11.1 mM in distilled water.

Acetylcholine solution: The stock solution of acetylcholine was prepared in 5% $NaHPO_4$ solution so as to contain 10 mg/ml and it was stored at -4°C. Serial dilutions of 1 mg/ml, 100 mg/ml, 10 mg/ml, 1 mg/ml, and 100 ng/ml were prepared from the stock solution at the time of the experiment.[12]

Effect of free radicals on nicotinic and muscarinic receptor function

Effect of ACh in the presence of 100 mM H_2O_2 and hydroxyl free radicals on frog rectus abdominis (FRA): Frog (*Rana tigirina*) was pithed and the rectus abdominis muscle was dissected as thin as possible and placed in a petri dish containing frog Ringer solution. A small piece of the above tissue was mounted in an isolated organ bath containing frog Ringer's solution at room temperature. The tissue was maintained under a constant load of 1 g in 10 ml organ bath. It was equilibrated for 30 min with regular change of bath fluid every 10 min.[13] Dose response was recorded on the kymograph. After stabilization period of 10 min, the Ringer solution was replaced with Ringer solution containing ROS 100 mM H_2O_2 or Fm and each tissue was bathed for 10 min following which the submaximal dose response was recorded. In the same tissue, Ringer solution containing ROS was replaced with normal frog Ringer solution and submaximal dose response was recorded.

Effect of pretreatment with AA (antioxidant) on ACh in the presence of 100 mM H_2O_2 and Fm on FRA: The dose response curve was taken in the presence of frog Ringer solution containing AA (300 μg/ml) after remaining in contact with the muscle for 10 min. After that, the frog Ringer solution containing AA was replaced with Ringer solution containing 100 mM H_2O_2 or Fm. The rectus abdominis muscle was bathed in the above-mentioned solution for 10 min. Then, the responses were recorded.

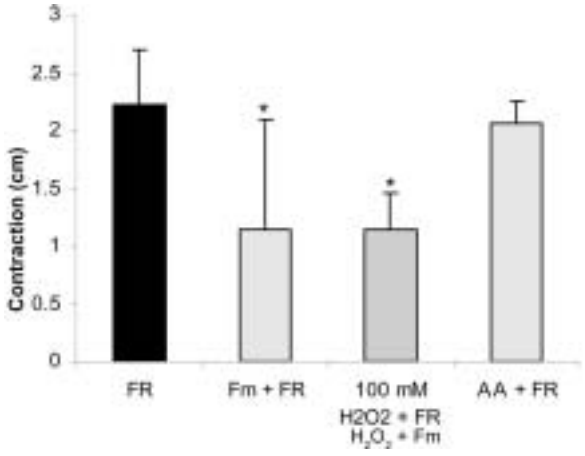
Effect of ACh in the presence of 100 mM H_2O_2 followed by treatment with AA on FRA: The dose response curve was recorded in normal frog Ringer solution. After that, the frog Ringer solution was replaced with frog Ringer solution containing 100 mM H_2O_2 and the rectus abdominis muscle was bathed in the above-mentioned solution for 10 min and the responses were recorded. Then, the above solution was replaced with frog Ringer solution containing AA and responses were recorded.

Effect of ACh diluted in 100 mM H_2O_2 and Fm on FRA: The dose response curve was recorded on FRA with ACh diluted in frog Ringer solution, 100 mM H_2O_2 and Fm separately and compared with dilutions of ACh in frog Ringer solution.

Effect of free radicals on muscarinic receptor function

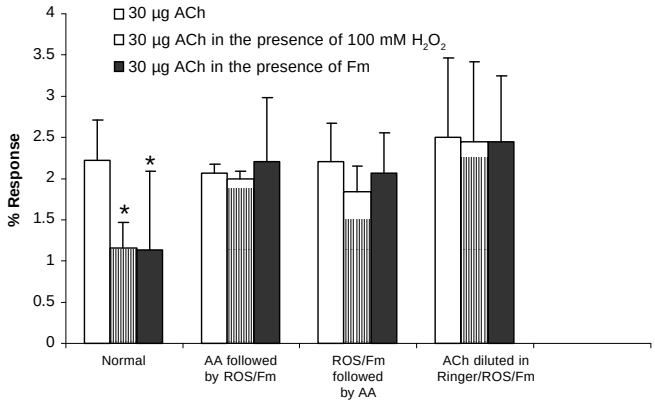
Effect of ACh in the presence of 1 mM H_2O_2 and Fm separately on isolated frog heart: Frog (*R. tigirina*) was pithed by conventional method and the heart was exposed. The pericardium was cut and a pair of threads was passed below the sinus venosus. The distal thread was tied and the proximal thread was made into a loop. After placing a forceps beneath it, a cut was made on the inferior vena cava towards the heart, and Syme's cannula inserted which in turn was connected to a reservoir of frog Ringer solution, and tied tightly with the help of proximal thread. After that, the heart was separated from other tissues and allowed to stabilize for about 10–15 min.[14] Cardiac output, heart rate, and the force of contraction were recorded with increasing concentrations of ACh (10, 30, and

Figure 1. Effect of acetylcholine and H_2O_2 on isolated frog rectus



Values are mean±SD. n=6 in each group. *P<0.0001 compared to frog ringer group. FR-frog Ringer; Fm-fenton mixture; AA-ascorbic acid

Figure 2. Effect of ROS on frog rectus abdominis



*P<0.001, ROS has decreased the response of ACh, when compared with their effect in the presence of normal frog Ringer and AA. ROS had no effect when diluted in ACh. AA-Ascorbic acid, ROS-Reactive oxygen species, ACh-Acetylcholine and Fm-Fenton mixture

100 ng). After a stabilization period of 10 min, frog Ringer solution was replaced with frog Ringer solution containing ROS 1 mM H_2O_2 and Fm separately. The heart was perfused for 10 min after which ACh responses were recorded in the presence and absence of ROS in frog Ringer solution on the kymograph.

Effect of pretreatment of AA on ACh in the presence of 1 mM H_2O_2 and Fm separately on isolated frog's heart: ACh response was recorded in the presence of frog Ringer solution containing AA (300 μg/ml) after stabilization of the heart for 10 min. After that, the frog Ringer solution containing AA was replaced with frog Ringer solution containing 1 mM H_2O_2 and Fm separately. After stabilization for 10 min, ACh responses were recorded on the kymograph.

Effect of ACh in the presence of 1 mM H_2O_2 followed by treatment with AA on isolated frog heart: ACh response was recorded in frog Ringer solution after stabilization of heart for 10 min. After that, the responses were recorded with frog Ringer solution containing 1 mM H_2O_2. Then, the above solution was replaced with frog Ringer containing AA and after stabilization for 10 min the ACh responses were recorded again.

Effect of ACh diluted in 1 mM H_2O_2 and Fm separately on isolated frog heart: 10, 30, 100, and 300 ng of ACh were prepared in frog Ringer solution, 1 mM H_2O_2 and Fm responses were recorded separately on isolated frog's heart and compared with dilutions of ACh in frog Ringer solution.

The study was carried out after clearance by the institutional animal ethics committee.

Statistical analysis

The data were analysed using one-way ANOVA and P values <0.05 were considered significant.

Results

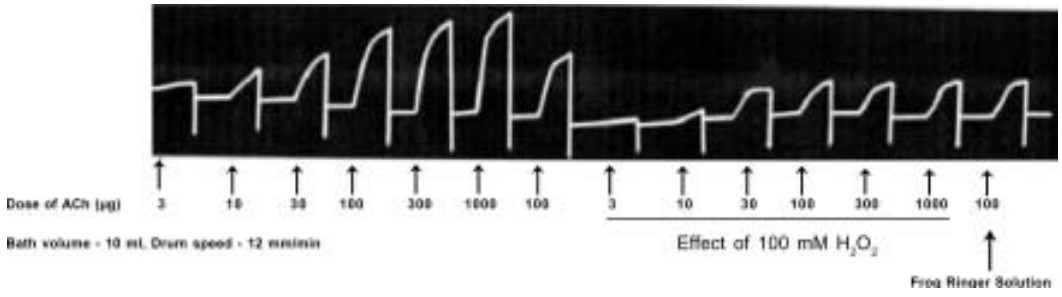
Effect of 100 mM H_2O_2 and Fm on nicotinic receptor function

From the dose response curve of ACh on FRA, the submaximal dose (30 μg) was selected. The mean percentage ±SD of the submaximal response was taken as 100%, which was considered as control. Reduction in the response with Ringer containing 100 mM H_2O_2 was 48.2% (P <0.001) compared with the control. The ACh response with frog Ringer containing 100 mM H_2O_2 before and after treatment with AA was not changed when compared with the control. There was 48.7% reduction in ACh response in the presence of Fm compared (P<0.001) with the control and the ACh response recovered with normal frog Ringer solution [Figure 1]. On pretreatment of FRA with AA, there was no reduction in ACh response with frog Ringer containing Fm. There was no change in ACh response when recorded with ACh prepared in frog Ringer solution, 100 mM H_2O_2 and Fm separately [Figures 2 and 3].

Effect of 1 mM H_2O_2 and Fm separately on muscarinic receptor function

There was a significant (P<0.001) decrease in the force of contraction (0.7±0.05) and increase in heart rate (74.3±9.54) in the presence of 1 mM H_2O_2 in frog Ringer plus 30 ng ACh as compared to the effect of frog Ringer solution alone or along with 30 ng ACh. However, no significant difference was observed between 1 mM H_2O_2 in frog Ringer solution and other parameters. There was a change observed in cardiac output but it was statistically nonsignificant [Table 1 and Figure 4]. There was a similar action with 1 mM H_2O_2 on isolated frog heart before and after treatment with AA at 30 ng of ACh when compared with frog Ringer solution alone. There was a significant decrease in the force of contraction and heart rate in frog Ringer solution containing Fm when compared with frog Ringer solution alone. There was a decrease in ACh response (30 ng) in the presence of Fm containing frog Ringer on comparison with ACh response in normal frog Ringer solution. ACh response was reversed on reperfusion with normal frog Ringer solution. On pretreatment of FRA with AA, there was a similar action with Fm on isolated frog heart. There was no change in ACh response when recorded with ACh

Figure 3. Effect of 100 mM hydrogen peroxide on frog rectus abdominis by using acetylcholine response.



The dose reponse curve of ACh on frog rectus abdominis followed by its effect in the presence of ROS. There is a significant decrease in the contraction of the rectus abdominis muscle in the presence of ROS.

Table 1

Effect of acetylcholine in the presence of 1 mM H_2O_2 on isolated frog heart

Parameters	*Force of contraction (cm)*	*Cardiac output (ml/min)*	*Heart rate/min*
Frog Ringer	1.82 ± 0.09	9.5 ± 1.23	43.5 ± 8.45
Frog Ringer + 30 ng ACh	0.1 ± 0.05	6.4 ± 1.45	34.9 ± 6.54
1 mm H_2O_2 in frog Ringer	1.62 ± 0.08	6.8 ± 1.89	75.1 ± 10.21
1 mm H_2O_2 in frog Ringer's + 30 ng ACh	0.7 ± 0.05*	6.2 ± 1.25	74.3 ± 9.54*

n = 6 in each group. *P <0.001; compared to 30 ng ACh in frog Ringer

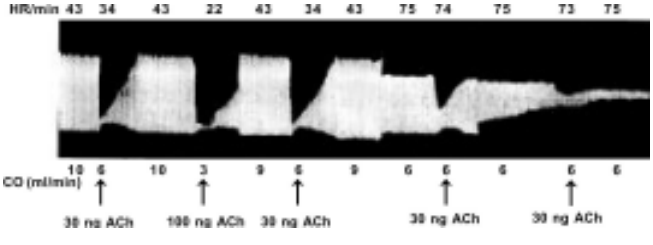
prepared in frog Ringer solution, 1 mM H_2O_2 and Fm.

Discussion

It is clearly evident that free radicals play a pivotal role in the etiology of many diseases. Local free radical formation that occurs under certain pathological conditions, like ischemic reflow, may regulate receptor response.[3] Free radical susceptibility of receptors can be deduced from the presence of functionally critical sulfhydryl group located in the receptor proteins.[15]

In the present study, it has been observed that the free radical generation and loss in receptor function were concentration-dependent, which has earlier been reported by Olszewski *et al*.[16] on trachealis muscle of horse, suggesting that more generation of the free radicals leads to greater receptor damage, which may ultimately result in reduced receptor function. Further, the concentration-dependent effect of ROS was also observed in both the nicotinic receptors of FRA and muscarinic receptors of isolated frog heart, which is thought to be due to the involvement of sulfhydryl groups; it has been proposed for muscarinic cholinergic receptors and detailed molecular delineation of the cysteine residues involved in disulfide bonding of the receptor protein was studied.[15] Ashkenazi *et al*. reported that ROS potentiate the negative inotropic and attenuate the positive inotropic signalling events in M_2 receptor, thereby potentiating the negative inotropic effect of muscarinic receptor agonist (ACh) in isolated rat left atria. These actions of the M_2 receptor are thought to be mediated by inducing phospholipase C-mediated phospoinositide turnover.[17]

Figure 4. Effect of 1 mM hydrogen peroxide on isolated frog heart by using acetylcholine response



The response of ACh on isolated frog heart in normal frog Ringer solution followed by its effect with ROS is shown. There is a significant decrease in the force of contraction of the heart and increased in heart rate.

Doleman *et al*., in 1988 reported the antioxidant effect of vitamin E and selenium in lung tissue after exposure to ROS[18] and Mangelus *et al*., in 2001 also reported that the Ras, extra cellular signal-regulated kinase and p38 are responsible for the imbalance which is brought about by muscarinic activation. These pathways were blocked by the antioxidant N-acetylcysteine when exposed to ROS.[9] In the present study on pretreatment of frog's rectus abdominis with AA, there was no reduction in ACh response with frog Ringer containing ROS. It is further evident that there is no effect of ROS on nicotinic receptor function on prior exposure to antioxidants (AA). In contrast, AA has not shown any beneficial effect on the muscarinic receptor site. The study on exposure of antioxidants after treatment with ROS showed that the receptor activity has been reversed in the nicotinic receptor in contrast to the

muscarinic receptor, which may be due to permanent loss of muscarinic receptor activity. This again implies that the muscarinic receptors are more susceptible to free radicals than the nicotinic receptors. It may be attributed to the sulfhydryl groups of the G-protein-coupled muscarinic receptors, which are more susceptible and sensitive to ROS than the ion-channel-linked nicotinic receptors.

The investigation also reveals that, with increase in exposure time (10–30 min) of ROS at the receptor site, there is a corresponding decrease in receptor activity even at a higher dose of ACh (300 μg). This clearly indicates that effective free radical attack is time dependent. The time-dependent effect of ROS was observed both in nicotinic and muscarinic receptors. The effect of ACh was reduced with very low concentration of ROS (1 mM H_2O_2, 1:50 Fm) on muscarinic receptors, compared to that on nicotinic receptors (0.1 M H_2O_2 or 3:50 Fm). This also indicates that the muscarinic receptors are more sensitive to ROS than nicotinic receptors.

Reactive oxygen species has shown different effects on receptor function. The actions of Fm were reversed both in nicotinic and muscarinic receptors when replacing with normal frog Ringer's solution but not with H_2O_2. This indicates that H_2O_2 causes permanent damage at the receptor site. ACh diluted in Fm and H_2O_2 showed no change in ACh response, which indicates that the effect of ROS was due to altered receptor function but not due to chemical alteration of ACh or *in vitro* interaction.

Conclusion

From the above study, it is clear that free radicals cause damage to the receptors. G-protein-coupled muscarinic receptors are more susceptible than ion-channel-linked nicotinic receptors. Ascorbic acid, a free radical scavenger, offers protection from ROS at the receptor site. A thorough and detailed investigation had been planned to support the study.

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