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Carbon Centered Radicals Reduce the Density of L-Type Calcium Channels in Rat Cardiac Membranes

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The carbon centered radicals generated by preincubation of cumene hydroperoxide with rat cardiac membranes dose dependently reduced the B_{max} of [^{3}H]nitrendipine binding, while K_D was unchanged. The reduction induced by cumene hydroperoxide was prevented by 2,6-di-t-butlyl-4-methyl-phenol. The generation of ·OH did not influence the [^{3}H]nitrendipine binding; also other oxidative agents (H_2O_2 and $HClO_4$) did not modify this binding. Therefore the reduction in [^{3}H]nitrendipine binding sites is not attributable to generic oxidative stress but to the formation of carbon centered radicals.

L-type calcium channels represent a major pathway by which Ca^{2+} gains entry into cardiac cells and therefore they play an important role in excitation contraction coupling in cardiac tissue (1–4). They can be characterized by the binding of organic calcium blockers of the 1,4 dihydropyridine class. (5–6). In the rat, ischemia followed by reperfusion (7) reduces 1,4 dihydropyridine binding, while ischemia alone does not modify it. Also in the guinea-pig heart, hypoxia and reoxygenation reduces the maximal number of binding sites (B_{max}) of [^{3}H]nitrendipine binding, while hypoxia alone does not produce any change (8). Furthermore the [^{3}H]nitrendipine binding to rat cardiac membranes is reduced by preincubation with xanthine oxidase (XOD), a system that generate oxygen free radicals (9,10,11). During reperfusion and reoxygenation it is well known that there is an increase in the production of free radicals (12,13,14), as happens when cardiac membranes are incubated with XOD (15,16). These results suggest that oxidative stress reduces dihydropyridine binding to cardiac membranes; it is still a matter of discussion which species of free radicals is involved. Therefore this study was performed to clarify this point.

MATERIALS AND METHODS

Membrane preparation. Adult male Sprague-Dawley rats (200–250 g) were sacrificed by decapitation. Hearts were excised and perfused through the aorta with 10 ml of cold saline solution (NaCl 0.9%) and cardiac membranes were prepared as previously described (8).

In brief, the procedure involves Ultra Turrax homogenization of tissue diluted 1:100 (wet weight/homogenizing volume) in 50 mM Tris-HCl, pH = 7.4 and filtration through four layers of gauze.

The resultant homogenate was centrifuged at 48000 × g for 10 min and the pellet was washed five times in the same buffer at the same speed. Resuspension of the final pellet was performed with Tris-HCl buffer (final tissue concentration 50 mg original weight/ml buffer). Before binding assays, crude membrane preparation was incubated with XOD (11), boiled XOD, cumene hydroperoxide (Cum00H) (17), $FeCl_2$ + $FeSO_4$ + H_2O_2 (18) and myeloperoxidase (MPO) + H_2O_2 (19) at the concentrations indicated in the results for 15 min at 37°C. In some experiments, the membranes were preincubated with 1 mM 2,6-di-*t*-butyl-4-methyl-phenol (BHT) (17) for 15 min at 37°C. Controls of each samples were preincubated and

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incubated as above without any reagent. After the incubation period the membrane suspension was washed in 3 ml of ice-cold buffer and centrifuged at 48000 × g for 10 min and used for the binding experiments.

For Electron-Spin Resonance (ESR) measurement, the five-washed pellet was resuspended to a final concentration of 50 mg original tissue weight/ml buffer and fixed aliquots (0.2 mg/200 ml) were supplemented with 0.1 M 5,5-dimethyl-1-pyrroline N-oxide (DMPO) and the mixture producing free radicals at the concentrations shown in the results.

Binding experiments. Ventricular membranes were incubated in a shaking water bath with [^{3}H]nitrendipine (0.01–1.25 nM) for 90 min at 25°C in a final volume of 2 ml 50 mM Tris-HCl pH = 7.4 (8). Binding was terminated by dilution with 3 ml of cold buffer and rapid vacuum filtration over Whatman GF/B filters, followed by three rinses with 5 ml of cold buffer; the trapped radioactivity was subsequently measured in 4 ml of Filter Count (Packard Instruments) by liquid scintillation spectrometry (Tri-Carb 1900TR, Packard Instruments). Non-specific binding was measured in the presence of 1 mM unlabelled nitrendipine. Competition curves were performed by a standard displacement technique using 0.05 nM [^{3}H]nitrendipine and increasing concentrations (0.001–1000 nM) of nifedipine.

The binding assay was performed in triplicate. Protein was determinated by the method of Bradford (20) using bovine serum albumin as standard. Because of the known photosensitivity of the dihydropyridines, the binding experiments were carried out in the dark.

ESR studies. To increase the signal-to-noise ratio and to allow measurements of the variation of signal intensity, the solutions were injected into a flat cell fixed in the ESR cavity and a flow and stop procedure was used to guarantee the recording of the spectra under the same experimental conditions. Freshly prepared solutions were used. However, each ESR experiment was repeated several times to control the reproducibility of the results.

The ESR spectra were recorded by means of a Bruker 200D spectrometer operating in the X band, interfaced with Stelar software to a PC-IBM computer for data acquisition and handling. The temperature was controlled with the aid of a Bruker ST 100/700 variable-temperature assembly. Magnetic parameters were measured by field calibration with the 2,2-di(4-tart-octylphenyl)-1-picrylhydrazyl (DPPH radical) (g = 2.0036).

Analysis of the experimental results. Saturation and competition data were evaluated quantitatively with non linear least square curve fittings using the computer programs LIGAND (20) and also fitted with the general dose-response equation (21).

Data are presented as means ± s.e. Statistical analysis was performed by Student's t test. P values < 0.05 were considered significant.

The ESR spectra were analyzed by measuring the hyperfine (hpf) constants, A_i, for the coupling between the unpaired electron spin and the nuclear spins of the nitrogen nucleus of DMPO-radical and of the surrounding protons in the radical structure. Each group of magnetically equivalent protons gave rise to a multiplet (n + 1, where n is the number of protons) of lines in the ESR spectra, and the hpf constants were affected by the overall structure of the radical. The A_i values were therefore distinctive of a type of radical. The spectra were simulated as a combination of lorentzian lines (the multiplets of lines arising from the hpf coupling). Integration of the adsorption signals allowed for the evaluation of the intensities of the spectra.

Usually DMPO produces radical species by aging or light exposure. In any case the signal from a water solution of DMPO was subtracted from the signals of the samples under study, recorded in the same experimental conditions.

Chemicals. [^{3}H]nitrendipine (70–87 Ci/mmol) was obtained from New England Nuclear Corporation (Boston, USA). Unlabelled nitrendipine and nifedipine were gifts from Bayer (Italy).

CumOOH and XOD were purchased from Sigma Chemical Company (St. Louis, USA) and BHT from Fluka Chemie A. G. (Buchs, Switzerland). DMPO was obtained from Aldrich Chimica (Italy).

All other chemicals were of highest purity available.

RESULTS

Binding experiments. Table 1 summarizes the results obtained when cardiac membranes were preincubated with different oxidative agents. The measured values of binding were unchanged by H_2O_2 or by a system generating hypochlorosus acid or by a system generating OH radicals. Pilot experiments showed that iron salt did not influence the binding (data not shown). On the other hand, XOD and CumOOH reduced it. The effect of CumOOH, a well known inhibitor of lipid peroxidation, was dose-dependent and its action was prevented by preincubation with BHT (table 2).

The Scatchard plots of saturation curves of [^{3}H]nitrendipine binding performed in membranes treated or not treated with CumOOH revealed that dissociation constant (K_D) was unaffected while the B_{max} was statistically reduced (fig 1). The displacement curve of [^{3}H]nitrendipine binding performed with nifedipine, indicated that CumOOH treatment did not modify the inhibition constant, being 1.54 ± 0.08 nM in control and 1.52 ± 0.27 nM in treated membranes (n = 5).

TABLE 1
Effect of Several Oxidative Agents on [^{3}H]Nitrendipine Binding Measured in Rat Cardiac Membranes

treatment	[fmol/mg protein]	*n*
Control	32.1 ± 1.3	3
25 mU XOD	19.4 ± 0.7*	3
25 mU boiled XOD	31.5 ± 1.8	3
Control	36.9 ± 3.7	11
1 mM H_2O_2	35.6 ± 3.3	9
32 mU MPO	38.4 ± 5.2	5
1 mM H_2O_2 +32 mU MPO	38.5 ± 3.7	5
Control	34.7 ± 1.2	5
1 mM H_2O_2	33.7 ± 1.8	5
1 mM H_2O_2 +0.2 mM Fe(II)/Fe(III)	33.4 ± 2.8	5

Membranes were preincubated for 15 min at 37°C plus the addition shown; controls were incubated under similar condition without any additions. XOD = xanthine oxidase; MPO = myeloperoxidase; [Fe(II)] = $FeSO_4$; [Fe(III)] = $FeCl_3$. Values are mean ± s.e. of *n* = number of experiments. * $P < 0.05$.

ESR studies. The free radical generation was monitored by an ESR technique. Figure 2 shows the ESR spectra recorded from 2.5 mM CumOOH homogenous mixture in water with 0.1 M DMPO in the absence (a) and in presence (b) of the cardiac membranes.

Apparently the shapes of the spectra were the same in the two conditions, but the different magnetic parameters (reported in the caption of fig 2) show that the main component of signal (a) arose from carboxy radicals, while the main component of signal (b) was due to methoxy radicals. Moreover the (b) signal was double the intensity of signal (a).

Small fractions of two other signals, due to trapping of ·OH and ·H radicals, were persistent in the two signals. These signals are not henceforth discussed.

Other experiments were carried out by using the Haber-Fenton reaction (23), which produced ·OH radicals from the redox system Fe(II)–Fe(III) in the presence of H_2O_2. The DMPO adduct gave rise to the well known four-line ESR signal characterized by $A_N = A_H$ (b proton) = 15.2 G (18). The addition of cardiac membrane did not produce any change in the spectral features and the signal intensity was reduced about ten times (data not shown).

TABLE 2
Dose Response Curve of CumOOH on [^{3}H]Nitrendipine Binding in Rat Heart Membranes and Protective Effects of BHT Preincubation

treatment	[fmol/mg protein]	*n*	*p*
Control	33.8 ± 2.5	6	
1 mM CumOOH	27.3 ± 1.6	6	N.S.
2.5 mM CumOOH	26.0 ± 0.9	6	<0.05
5 mM CumOOH	16.0 ± 2.2	6	<0.001
1 mM BHT + 2.5 mM CumOOH	33.4 ± 3.2	3	N.S.

Cardiac membranes were preincubated with BHT for 15 min and incubated with CumOOH for 15 min. Values are the means ± s.e. of different preparations, *n* = number of experiments; *p* = statistical analysis versus control values.

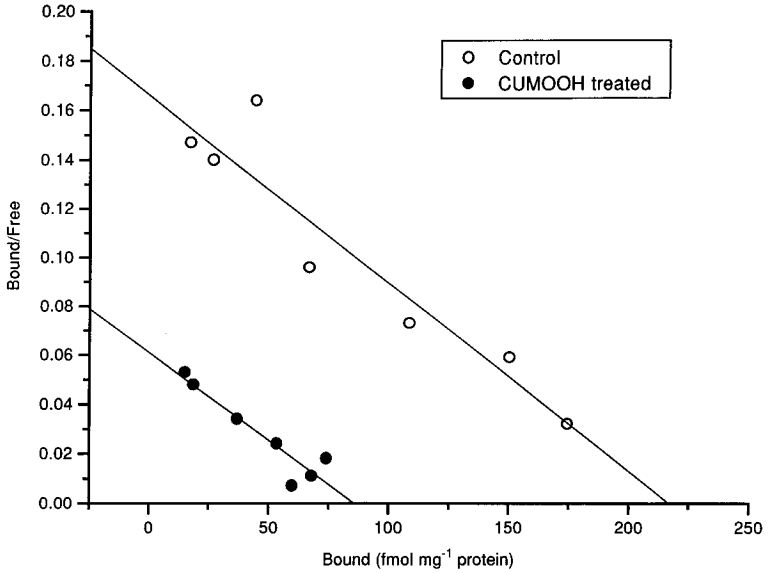

FIG. 1. Equilibrium binding of [^{3}H]nitrendipine to cardiac membranes preincubated for 15 min with or without 2.5 mM CumOOH. *Ordinate* B/F, bound to free ratio; *abscissa* concentration of bound ligand. [^{3}H]nitrendipine (from 0.01 to 1.25 nM) was incubated with cardiac membranes for 90 min at 25°C with or without nifedipine (1 μM) to measure non-specific binding. This plot was derived from the fit of two typical curves and each point is the mean of triplicate determinations.

DISCUSSION

The [^{3}H]nitrendipine binding is not modified by oxidative stress, but it is reduced only by XOD and CumOOH.

The hyperfine parameters evaluated from the spectra in Fig. 2 are consistent with the formation of two different types of radical from CumOOH in the absence and presence of the cardiac membrane. In particular, in the absence of the membrane, the methoxy-radical, that is, $CH_3 \cdot CHOH$, is mainly formed, whereas in the presence of the membrane twice the amount of the carboxy radical, that is, $\cdot COO^-$, is produced compared to the methoxy radical. This carbon centered radical is usually found in the presence of carboxylates (24). In the membrane, the lipid peroxidation induced by CumOOH may be responsible for the production of the carboxy radicals. This could explain the reduction in the [^{3}H]nitrendipine binding in the presence of CumOOH; moreover, BHT, a typical scavenger of carbon centered radicals, prevents the effect on [^{3}H]nitrendipine binding.

The carboxy radical has also been formed with XOD (11), and XOD preincubation also reduced the binding of L-type calcium channel antagonist.

It is well known that the reaction of Fe(II)–Fe(III) + H_2O_2 + DMPO results in generation of OH radicals, whose intensity is reduced in the presence of membrane (data not shown); this quenching may be due to a scavenger effect of the membrane. The addition of the ·OH-producing mixture to the membrane preparation causes no effect in the [^{3}H]nitrendipine binding and only a small amount of ·OH radicals remain in the presence of the membrane. These findings indicate that the ·OH radicals do not participate in the [^{3}H]nitrendipine binding process and that this process is strongly affected by the production of radicals, which have been identified as carbon centered radicals. These radicals reduce B_{max} without affecting the cognitive site of channels; in fact K_D and half maximal ineffective concentration (IC_{50}) values were very similar to those observed in control membranes.

The above results suggest that [^{3}H]nitrendipine binding is influenced by carboxy radicals; and it seems likely that CumOOH, which is known to affect cardiac function (25), could exert its toxicity throughout this mechanism.

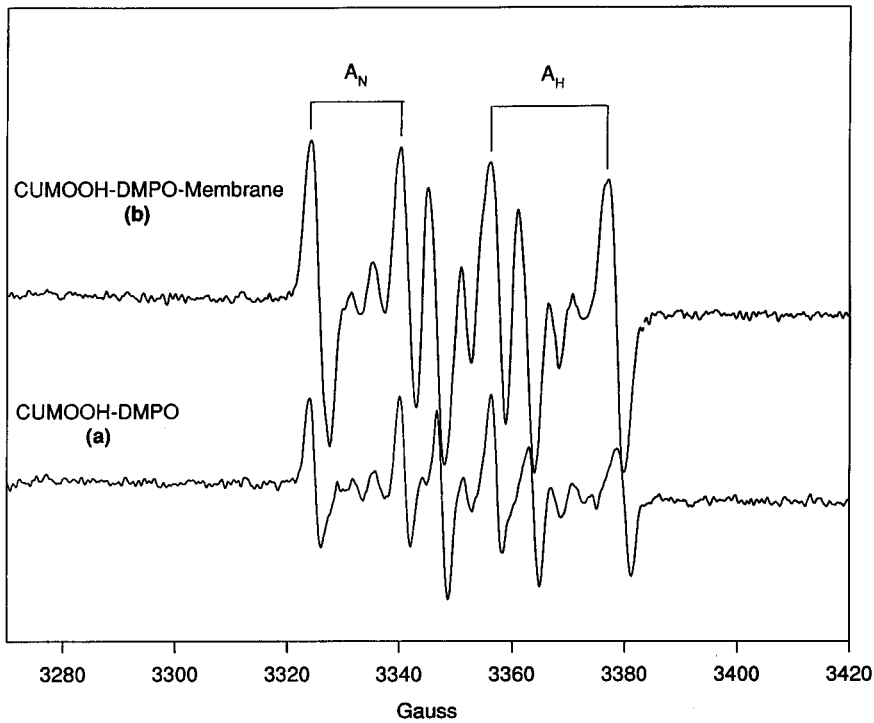

FIG. 2. ESR spectra of CumOOH (2.5 mM)-DMPO (0.1 M) homogeneous mixture in the absence (a) and in the presence (b) of cardiac membranes (0.2 mg/200 ml). (a) A_N = 16.3 G; A_H (b proton) = 22.6 G. (b) A_N = 15.8 G; A_H (b proton) = 19.0 G.

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