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Effects of CP-060S, a novel Ca^{2+} channel blocker, on oxidative stress in cultured cardiac myocytes

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Abstract

The effect of (−)-(*S*)-2-[3,5-bis(1,1-dimethylethyl)-4-hydroxyphenyl]-3-[3-[*N*-methyl-*N*-[2-(3,4-methylenedioxyphenoxy)-ethyl]amino]propyl]-1,3-thiazolidin-4-one hydrogen fumarate (CP-060S), a novel Ca^{2+} channel blocker, on hydrogen peroxide (H_2O_2)-induced cytotoxicity was studied in cultured rat cardiac myocytes. The CP-060S effect was compared with that of CP-060R, an optical isomer of CP-060S with a less potent Ca^{2+} channel blocking action than CP-060S. H_2O_2 increased the release of lactate dehydrogenase from cardiac myocytes and decreased the formation of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) (MTT) formazan in cardiac myocytes (i.e., cytotoxic action). Both CP-060S (1 μM) and CP-060R (1 μM) attenuated to a similar extent the foregoing alterations induced by H_2O_2. On the otherhand, 1,3-dimethyl-2-thiourea (10 mM), a scavenger of both H_2O_2 and hydroxyl radical, also attenuated the H_2O_2-induced cytotoxicity whereas diltiazem (10 μM) did not. In an experiment using electron spin resonance (ESR) with 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO), a spin-trapping agent, both CP-060S and CP-060R decreased the intensity of DMPO-hydroxyl radical signal concentration dependently. These results suggest that CP-060S protects cardiac myocytes from oxidative stress through its radical scavenging action.

Keywords: Anti-oxidant; Ca^{2+} channel blocker; Cardiac myocyte; Diltiazem; Hydrogen peroxide

1. Introduction

Recently, (−)-(*S*)-2-[3,5-bis(1,1-dimethylethyl)-4-hydroxyphenyl]-3-[3-[*N*-methyl-*N*-[2-(3,4-methylenedioxyphenoxy)ethyl]amino]propyl]-1,3-thiazolidin-4-one hydrogen fumarate (CP-060S) (Fig. 1) has been demonstrated to protect the myocardium from ischemia–reperfusion damage in animal models (Koga et al., 1998; Suzuki et al., 1998b). For example, the compound inhibits the arrhythmia induced by ischemia and reperfusion in rats (Koga et al., 1998) and reduces infarct size in the heart subjected to ischemia and reperfusion in dogs (Suzuki et al., 1998b). One of the characteristic pharmacological properties of CP-060S is a potent blocking action on the L-type Ca^{2+} channel in cardiac and vascular smooth muscles (Ohya et al., 1997; Suzuki et al., 1998a). In guinea-pig mesenteric artery, the Ca^{2+} channel blocking action of CP-060S is about 30 times more potent than that of diltiazem, which is widely used for treatment of ischemic heart disease (Ohya et al., 1997). Therefore, one of the primary mechanisms of the cardioprotective action of CP-060S would be the improvement of the myocardial oxygen balance between supply and demand in the ischemic heart. This could be done by either increasing coronary flow or decreasing cardiac mechanical function, or both, resulting from the Ca^{2+} channel blocking action.

During ischemia and reperfusion, reactive oxygen species such as superoxide anion, hydroxyl radical, and hydrogen peroxide (H_2O_2), are generated intra- and extracellularly in the myocardium and endothelium (Hess and Manson, 1984; Lucchesi, 1990; Loesser et al., 1991). These reactive oxygen species cause lipid peroxidation of the cell membrane and intracellular Ca^{2+} overload, and hence cause myocardial derangements, including arrhyth-

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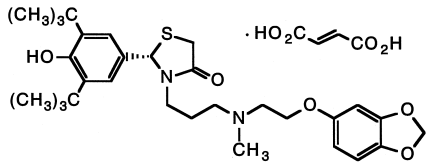

Fig. 1. Chemical structure of CP-060S.

mia and necrosis (Hess and Manson, 1984; Lucchesi, 1990; Loesser et al., 1991). In fact, some radical scavengers or anti-oxidants are effective in attenuating ischemia–reperfusion damage (Hess and Manson, 1984; Lucchesi, 1990; Loesser et al., 1991). Interestingly, a component of the chemical structure of CP-060S [3,5-bis(1,1-dimethylethyl)-4-hydroxyphenyl group] (i.e., a phenolic structure) is similar to that of phenol-based free radical scavengers, such as vitamin E and probucol. The phenolic structure is responsible for a radical scavenging activity (Halliwell and Gutterridge, 1989; Gotoh et al., 1992), and therefore CP-060S may protect the myocardial cell against oxidative stress. If CP-060S attenuates the cardiac damage induced by oxidative stress, the action of the compound to attenuate the oxidative stress may contribute to its protective action against ischemia–reperfusion damage to the myocardium. The beneficial action of many compounds against oxidative stress has been demonstrated in cultured cardiac myocytes (Janero et al., 1991; Byler et al, 1994; Horwitz and Leff, 1995; Horwitz et al., 1996), in which the direct effect of drugs on the cardiac cell can be demonstrated.

In the present study, therefore, the effect of CP-060S on the cytotoxicity induced by H_2O_2 was studied in cultured rat cardiac myocytes, and compared with the effect of CP-060R, an optical isomer of CP-060S having a less potent Ca^{2+} channel blocking action than CP-060S (Tamura et al., 1996) (the first series of experiments). We also examined whether CP-060S and CP-060R have radical scavenging actions (the second series of experiments).

2. Methods

The protocol of animal experiments in the present study was approved by the Asahikawa Medical College Committee on Animal Research.

2.1. Primary culture of cardiac myocytes (the first series of experiments)

Primary cultures of cardiac myocytes were prepared according to minor modifications of the methods of Suzuki et al. (1989, 1997). Briefly, 10 to 20 hearts isolated from neonatal Sprague–Dawley rats (2–3 days old) were immediately placed in a HEPES-buffered salt solution (HBSS) containing (in mM) HEPES 30, NaCl 120.5, KCl 2.0, KH_2PO_4 1.0 and glucose 4.1 (pH 7.6). The hearts were rinsed with HBSS to remove blood, minced into fine pieces in HBSS, and dissociated into cells with 10 ml of HBSS containing 0.1% collagenase for 45 min at 37°C. The isolated heart cells were washed three times with HBSS, and then the cell suspension was passed through a stainless mesh (45 μm) to remove cell aggregates. The cells were resuspended in a culture medium (F-12 supplemented with 15 mM HEPES, 10 μg/ml insulin, 10 μg/ml transferrin and 10 ng/ml sodium selenite). To separate the cardiac myocytes from non-myocytes (e.g., endothelial and fibroblastic cells), the cell suspension was incubated in culture flasks in a culture medium supplemented with 5% heat-inactivated fetal bovine serum. After incubation for 60 min, cells unattached to the culture flasks (myocytes) were passed through a stainless mesh (30 μm), and then washed with HBSS. The purified cardiac myocytes were finally resuspended in the serum-free culture medium to minimize the influence of anti-oxidants in the fetal bovine serum and the proliferation of non-myocytes. The cell suspensions were plated in fibronectin-coated 24-well culture plates (Nunc) at a density of 2.5×10^5 cells/ml per well. The cardiac myocytes were cultured in an incubator (APMW-36; ASTEC) with 5% CO_2 and 95% air at 37°C. The culture medium is replaced by 1 ml of fresh culture medium every 2 days.

2.2. Experimental protocols (the first series of experiments)

The effect of CP-060S, CP-060R, diltiazem and 1,3-dimethyl-2-thiourea, a scavenger of both H_2O_2 and hydroxyl radical (Jackson et al., 1985), on the H_2O_2 (150 μM)-induced cytotoxicity was studied in cultured cardiac myocytes. After 7 to 8 days of primary culture, the culture medium in each well was replaced by 1 ml of the culture medium containing CP-060S (0.1 or 1 μM), CP-060R (0.1 or 1 μM), diltiazem (10 μM), or 1,3-dimethyl-2-thiourea (10 mM). After 30 min upon replacement, 7.65 mM H_2O_2 (20 μl) was added to the culture medium containing these same agents so that the final concentration of H_2O_2 in the culture medium would be 150 μM. Therefore, the concentration of CP-060S and CP-060R changed from 0.1 to 0.098 μM, or from 1 to 0.98 μM when H_2O_2 (20 μl) was added to the culture medium, because the total volume of the culture medium increased from 1.0 ml to 1.02 ml during the H_2O_2 exposure. Similarly, the concentration of diltiazem and that of 1,3-dimethyl-2-thiourea changed from 10 to 9.8 μM and from 10 to 9.8 mM, respectively, when H_2O_2 (20 μl) was added to the culture medium. After 0.5, 1.5 and 3 h from the start of H_2O_2 exposure, cytotoxicity was assessed by measuring the release of lactate dehydrogenase (LDH) from the cardiac myocytes and the formation of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) formazan in cardiac myocytes.

A preliminary experiment was carried out to determine the effect of various concentrations of H_2O_2 (50, 150 or 300 μM) on viability of cultured cardiac myocytes. The LDH release from the cells and formation of MTT formazan were used as a marker of viability, and was measured at 0.5, 1.5 and 3 h after the start of the H_2O_2 exposure.

2.3. Determination of LDH release (the first series of experiments)

To evaluate the integrity of the sarcolemmal membrane, the amount of LDH released from cardiac myocytes was measured spectrophotometrically according to an enzymatic method (Bergmeyer, 1974), using an LDH assay kit (Sigma Chemical, St. Louis, MO, USA). For measurement of the LDH release, the culture medium (100 μl) was removed from each well at 0.5, 1.5 and 3 h after the start of the exposure to H_2O_2. Finally, the cells were completely lysed with 1% Triton X-100 to measure the amount of total (intra- and extracellular) LDH. The amount of LDH released from the cells (extracellular LDH) was expressed as a percentage of the total amount of LDH.

2.4. Determination of MTT formazan formation (the first series of experiments)

To determine the viability of cardiac myocytes, we carried out the MTT assay according to modifications of the method described by Mosmann (1983). The MTT assay is based on the ability of mitochondria to reduce MTT (a yellow tetrazolium dye) to MTT formazan (a blue mitochondrial by-product); the reduction is mediated by mitochondrial dehydrogenases in living but not in dead cells (Mosmann, 1983). This assay therefore may be one of the most appropriate ways to assess the viability of mitochondria-rich cells, including cardiac myocytes. At 0.5, 1.5 or 3 h after the start of the H_2O_2 exposure, the culture medium in each well was replaced by 1 ml of the mixture of MTT solution (5 mg/ml phosphate buffer) and F-12 (1:5) and incubated for 1 h at 37°C. After removal of the MTT-containing mixture, 1 ml of dimethyl sulfoxide (DMSO) was added to each well to dissolve MTT formazan, and the amount of MTT formazan in DMSO was then measured at a wavelength of 510 nm, using a spectrophotometer (V-520; JASCO, Tokyo, Japan). The amount of MTT formazan in H_2O_2-treated myocytes was expressed as a percentage of that in H_2O_2-untreated (normal) myocytes.

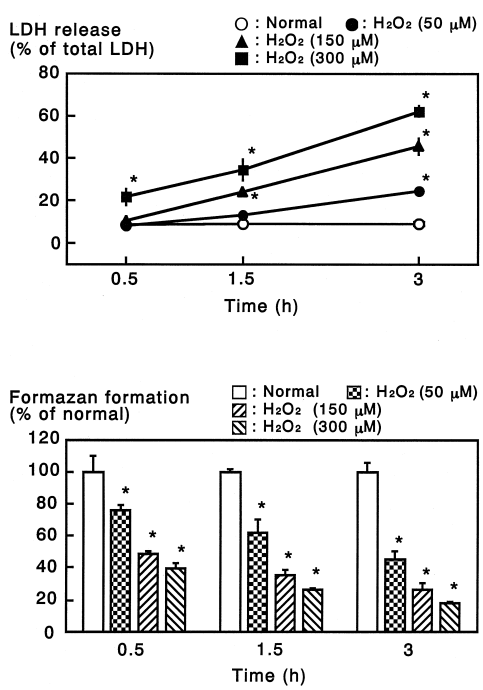

Fig. 2. Effects of various concentrations of H_2O_2 (50, 150 and 300 μM) on release of LDH from cardiac myocytes (upper panel) and formation of MTT formazan (bottom panel) in cardiac myocytes. Each value represents the mean ± SEM ($n = 4$). $^*P < 0.05$ when compared with value for the normal group.

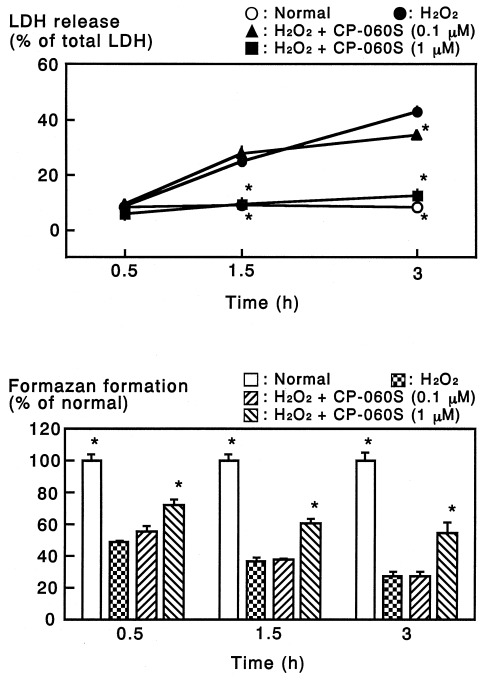

Fig. 3. Effects of CP-060S (0.1 and 1 μM) on the H_2O_2-induced changes in the LDH release from cardiac myocytes (upper panel) and the formation of MTT formazan (bottom panel) in cardiac myocytes. Each value represents the mean ± SEM ($n = 5$–12). $^*P < 0.05$ when compared with value for the H_2O_2 group.

2.5. Evaluation of H_2O_2-scavenging effect (the second series of experiments)

To determine whether CP-060S and CP-060R have a direct H_2O_2-scavenging effect, the effect of CP-060S and CP-060R on the H_2O_2 concentration was investigated in

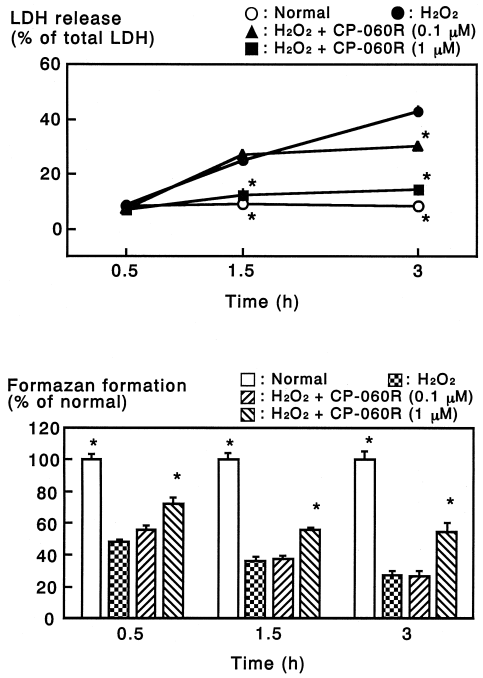

Fig. 4. Effects of CP-060R (0.1 and 1 μM) on the H_2O_2-induced changes in the LDH release from cardiac myocytes (upper panel) and the formation of MTT formazan (bottom panel) in cardiac myocytes. Each value represents the mean ± SEM. ($n = 6–12$). $^*P < 0.05$ when compared with value for the H_2O_2 group.

vitro and compared with that of 1,3-dimethyl-2-thiourea. The mixture of CP-060S (1 μM), CP-060R (1 μM) or 1,3-dimethyl-2-thiourea (10 mM) and H_2O_2 (150 μM) in the 0.1 M phosphate buffer (pH 7.0) was incubated at 37°C for 30 min. The concentration of H_2O_2 in the buffer was measured spectrophotometrically (Hayashi et al., 1989).

2.6. Evaluation of hydroxyl radical scavenging effect (the second series of experiments)

To estimate the scavenging ability of CP-060S and CP-060R against hydroxyl radicals, we used an electron spin resonance (ESR)-spin trapping method (Nakamura et al., 1997). Hydroxyl radicals were produced from Fe(II)-ion-catalyzed decomposition of H_2O_2, and 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO) was used as a spin trapping agent. The reaction mixture contained 20 μM DMPO, 150 μM H_2O_2 and 75 μM $FeCl_2$ in 50 mM potassium phosphate buffer, pH 7.0. The concentration of CP-060S and CP-060R in the reaction mixture was 1, 2.5 or 5 μM. ESR spectra of the reaction mixture were recorded at room temperature using a JES-TE300 ESR Spectrometer (JEOL, Tokyo, Japan) with 100 kHz modulation.

2.7. Reagents

The following reagents were used in the present study: CP-060S, CP-060R (Chugai Pharmaceutical, Shizuoka, Japan), diltiazem hydrochloride, insulin, transferrin, sodium selenite, DMPO (Sigma), 1,3-dimethyl-2-thiourea (Aldrich Chemical, St. Louis, USA), H_2O_2, MTT (Nacalai Tesque, Kyoto, Japan), F-12 (Cosmo Bio, Tokyo, Japan), collagenase (Wako Chemical, Osaka, Japan), and fetal bovine serum (Boehringer Mannheim, Germany).

CP-060S and CP-060R were dissolved in 0.01 N HCl and then diluted with the culture medium (the first series of experiments) or buffer solution (the second series of experiments) so that the final concentration of HCl in the culture medium or buffer solution is 0.1 mN. Other reagents were dissolved or diluted with the culture medium or buffer solution.

2.8. Statistical analysis

All values are expressed as mean ± SEM. When changes in LDH release in vehicle-treated (control) and drug-treated groups were to be compared, statistical analysis was performed with a two-way repeated measures analysis of variance (ANOVA) followed by Dunnett's test for multiple comparisons. If a significant difference was obtained between these groups, further comparisons at each time point were performed with Dunnett's test (Figs. 2–5). When the amounts of MTT formazan and the H_2O_2 concentration in control and drug-treated groups were to be compared, statistical analysis was performed with one-way ANOVA followed by Dunnett's test for multiple compar-

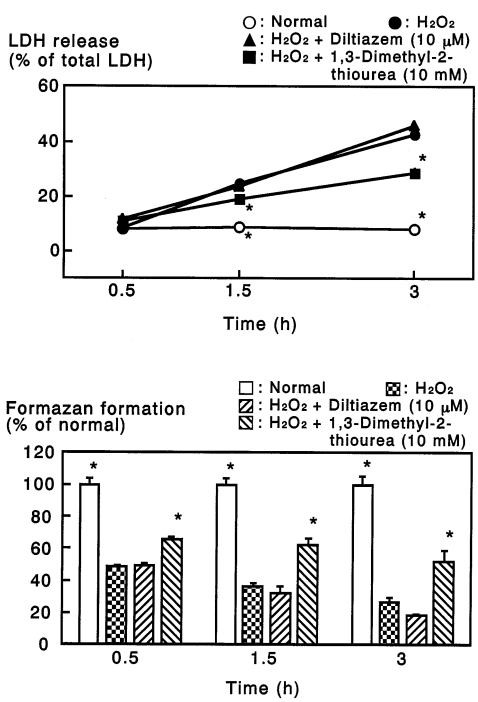

Fig. 5. Effects of diltiazem (10 μM) and 1,3-dimethyl-2-thiourea (10 mM) on the H_2O_2-induced changes in the LDH release from cardiac myocytes (upper panel) and the formation of MTT formazan (bottom panel) in cardiac myocytes. Each value represents the mean ± SEM ($n = 4–9$). $^*P < 0.05$ when compared with value for the H_2O_2 group.

Table 1
Effect of CP-060S, CP-060R and 1,3-dimethyl-2-thiourea on the concentration of H_2O_2 in the buffer solution.
Values are the means ± SEM of five samples for each experiment. The mixture of CP-060S (1 μM), CP-060R (1 μM) or 1,3-dimethyl-2-thiourea (10 mM), and H_2O_2 (150 μM) in the 0.1 M phosphate buffer (pH 7.0) was incubated at 37°C for 30 min. The H_2O_2 concentration in the buffer was measured spectrophotometrically.

Mixture	H_2O_2 concentration (measured)
H_2O_2 (150 μM)	149.1 ± 0.9
H_2O_2 (150 μM) + CP-060S (1 μM)	150.7 ± 0.4
H_2O_2 (150 μM) + CP-060R (1 μM)	151.3 ± 0.4
H_2O_2 (150 μM) + 1,3-dimethyl-2-thiourea (10 mM)	24.2 ± 0.4[a]

[a] $P < 0.05$ when compared with the value for the H_2O_2 (150 μM) group.

isons (Figs. 2–5, Table 1). A difference was considered statistically significant at $P < 0.05$.

3. Results

3.1. H_2O_2-induced cytotoxicity (the first series of experiments)

A preliminary experiment was performed to determine the extent of cellular damage induced by various concentrations of H_2O_2. Fig. 2 shows changes in LDH release and formation of MTT formazan induced by H_2O_2 at a concentration of 50, 150 or 300 μM. Also, H_2O_2 increased LDH release, and decreased the formation of MTT formazan in a concentration- and time-dependent way. After 3 h from the start of H_2O_2 exposure, the amounts of LDH released in 50, 150 and 300 μM H_2O_2-treated myocytes were about 24%, 46% and 62% of the total amounts of LDH, respectively. The amounts of MTT formazan in 50, 150 and 300 μM H_2O_2-treated myocytes were about 45%, 26% and 18% of that in H_2O_2-untreated (normal) myocytes, respectively. We therefore used 150 μM H_2O_2, which inflicts moderate to severe damage on cardiac myocytes, for the first series of experiments.

3.2. Effects of CP-060S and CP-060R on H_2O_2-induced cytotoxicity (the first series of experiments)

Figs. 3 and 4 show the effects of CP-060S and CP-060R, respectively, on the H_2O_2 (150 μM)-induced changes in LDH release and formation of MTT formazan. The release of LDH induced by H_2O_2 was almost completely inhibited by CP-060S (1 μM) and CP-060R (1 μM). Nevertheless, a low concentration of CP-060S (0.1 μM) and CP-060R (0.1 μM) failed to attenuate the H_2O_2-induced LDH release, except for 3 h after the start of the H_2O_2 exposure. CP-060S (1 μM) and CP-060R (1 μM) also attenuated the H_2O_2-induced decrease in formation of MTT formazan during the whole course of the study, although a low concentration of CP-060S (0.1 μM) or CP-060R (0.1 μM) did not attenuate it. In normal (H_2O_2-untreated) myocytes, neither CP-060S (1 μM) nor CP-060R (1 μM) affected the LDH release and formation of MTT formazan (data not shown). These results suggest that CP-060S and CP-060R protect cardiac myocytes from the H_2O_2-induced cytotoxicity.

3.3. Effects of diltiazem and 1,3-dimethyl-2-thiourea on H_2O_2-induced cytotoxicity (the first series of experiments)

Fig. 5 shows the effects of diltiazem and 1,3-dimethyl-2-thiourea on the H_2O_2 (150 μM)-induced changes in LDH release and formation of MTT formazan. Diltiazem (10 μM) did not attenuate the H_2O_2-induced changes in LDH release and formation of MTT formazan during the whole course of the study. In contrast, 1,3-dimethyl-2-thiourea (10 mM) attenuated the H_2O_2-induced changes in LDH release and formation of MTT formazan. In normal (H_2O_2-untreated) myocytes, neither diltiazem (10 μM) nor 1,3-dimethyl-2-thiourea (10 mM) affected the LDH release and formation of MTT formazan (data not shown). These results suggest that 1,3-dimethyl-2-thiourea, but not diltiazem, is effective in attenuating the H_2O_2-induced cytotoxicity.

3.4. Direct action of CP-060S and CP-060R on H_2O_2 or on hydroxyl radicals (the second series of experiments)

To examine whether there is a scavenging effect of CP-060S and/or CP-060R on H_2O_2, the concentration of H_2O_2 in the buffer solution in the presence or absence of CP-060S, CP-060R or 1,3-dimethyl-2-thiourea was measured (Table 1). 1,3-Dimethyl-2-thiourea decreased the H_2O_2 concentration markedly, whereas CP-060S or CP-060R did not. These results suggest that neither CP-060S nor CP-060R has a direct scavenging effect on H_2O_2.

The ESR spectra of DMPO-hydroxyl radicals in the presence or absence of CP-060S or CP-060R are shown in Fig. 6. The intensity of DMPO-hydroxyl radical signals decreased as the concentration of CP-060S or CP-060R increased. The second-order rate constant for the reaction of hydroxyl radicals with CP-060S or CP-060R was estimated to be 1.4×10^{10} M^{-1} s^{-1} from the kinetics based on the rate constant for the reaction of hydroxyl radicals with DMPO (3.4×10^9 M^{-1} s^{-1}) (Finkelstein et al.,

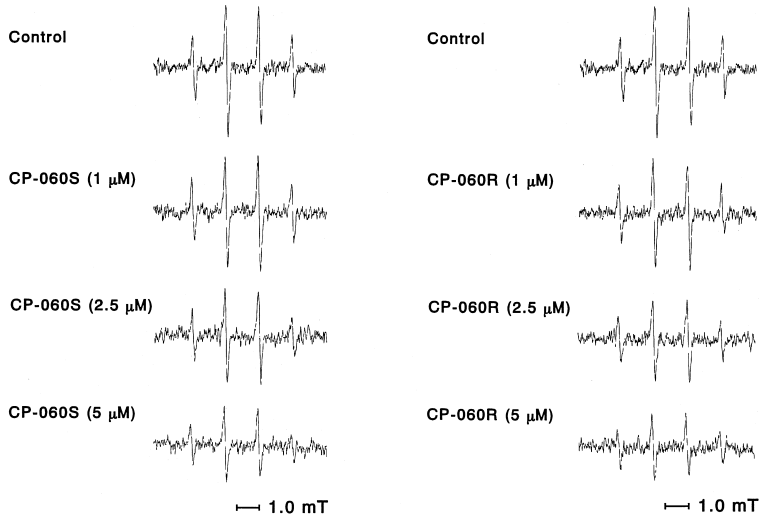

Fig. 6. Effect of CP-060S (1, 2.5 and 5 μM) and CP-060R (1, 2.5 and 5 μM) on the signal intensity of the DMPO-hydroxyl radical adduct.

1980). The results suggest that both CP-060S and CP-060R have a scavenging effect on hydroxyl radicals. Nevertheless, neither CP-060S nor CP-060R modified the intensity of DMPO-hydroxyl radical signals at a concentration of 1 μM, which was capable of attenuating the H_2O_2-induced cytotoxicity in the first series of experiments. DMPO competes with CP-060S or CP-060R for trapping of the hydroxyl radical, and therefore the required concentrations of CP-060S and CP-060R for scavenging the hydroxyl radicals could vary depending on the concentration of DMPO.

4. Discussion

Reactive oxygen species, such as superoxide anion, hydroxyl radical and H_2O_2, are considered to be important factors inducing myocardial damage during ischemia and reperfusion (Hess and Manson, 1984; Lucchesi, 1990; Loesser et al., 1991). Therefore, the action of substances that protect the myocardial cells from oxidative stress has been much studied (Janero et al., 1991; Byler et al., 1994; Clague and Langer, 1994; Horwitz and Leff, 1995; Horwitz et al., 1996). In the present study, the effect of CP-060S, a novel Ca^{2+} channel blocker, on oxidative stress was studied in cultured cardiac myocytes, and compared with the effect of CP-060R, an optical isomer of CP-060S. The Ca^{2+} channel blocking action of CP-060R is about eight times less potent than that of CP-060S in rat thoracic aorta (Tamura et al., 1996). We chose H_2O_2 as a reactive oxygen species for two reasons. First, H_2O_2 and its metabolite, hydroxyl radical, are considered important in the pathogenesis of myocardial damage induced by ischemia and reperfusion (Brown et al., 1988; Slezak et al., 1995). Second, H_2O_2 penetrates the cell membrane and reaches the intracellular site (Fisher, 1988), and therefore may produce severe damage to the cell.

H_2O_2 increased the LDH release from cardiac myocytes (i.e., impairment of sarcolemmal membrane) and decreased the formation of MTT formazan (i.e., impairment of mitochondrial activity) in cardiac myocytes, suggesting that H_2O_2 produces a cytotoxic action. The alterations in myocytes induced by H_2O_2 (LDH release and formation of MTT formazan) were significantly attenuated by CP-060S (1 μM) and CP-060R (1 μM), indicating that both CP-060S and CP-060R protect cardiac myocytes from the H_2O_2-induced cytotoxicity. It should be noted that the protective action of CP-060S and that of CP-060R on the H_2O_2-induced cytotoxicity were equivalent, and that 10 μM diltiazem, which is capable of blocking the L-type Ca^{2+} channel in cardiac myocytes (Klitzner et al., 1991), was ineffective in attenuating the H_2O_2-induced cytotoxicity. It is unlikely, therefore, that the Ca^{2+} channel blocking action is a major mechanism for the beneficial action of CP-060S on H_2O_2-induced cytotoxicity. This view gains support from findings that Ca^{2+} channel blockers such as diltiazem (Horwitz et al., 1996) and nifedipine (Clague and Langer, 1994) fail to attenuate the H_2O_2-induced cytotoxicity in cultured cardiac myocytes. Clague and Langer (1994) suggested that, in cultured cardiac myocytes, the L-type Ca^{2+} channel is not a major pathway for intracellular Ca^{2+} overload, which is responsible for the H_2O_2-induced cellular damage. Alternatively, activation of the Ca^{2+} leak channel (Clague and Langer, 1994), enhancement of Na^+/Ca^{2+} exchange (Goldhaber, 1996), inactivation of Ca^{2+}-adenosine triphosphatase (Kaneko et al., 1989), and an increase in membrane permeability (Hess and Manson, 1984) have been proposed as possible mechanisms of the H_2O_2-induced intracellular Ca^{2+} overload and myocardial damage.

According to biochemical studies, a hydroxyl radical generated from H_2O_2 by the Fenton reaction easily removes a hydrogen atom from a methylene carbon of an unsaturated fatty acid of the membrane phospholipids, and

hence initiates peroxidation of lipids (Hess and Manson, 1984). It has been demonstrated that lipid peroxidation of the cell membrane induced by reactive oxygen species is accompanied by intracellular Ca^{2+} overload and cellular damage in the myocardium (Hess and Manson, 1984, Nakaya et al., 1987). Some radical scavengers have been known to reduce the cytotoxicity induced by reactive oxygen species. For example, catalase (a scavenger of H_2O_2) (Horwitz and Leff, 1995) and DMSO (a scavenger of hydroxyl radical) (Byler et al., 1994) are effective in attenuating the H_2O_2-induced cytotoxicity in cultured cardiac myocytes. In the present study, 1,3-dimethyl-2-thiourea, a scavenger of both H_2O_2 and hydroxyl radical (Jackson et al., 1985), attenuated the H_2O_2-induced increase in LDH release and decrease in formation of MTT formazan. There is a possibility, therefore, that CP-060S and CP-060R scavenge H_2O_2, the hydroxyl radical or both, and hence attenuate H_2O_2-induced cytotoxicity. To test this possibility, we investigated whether CP-060S and CP-060R have a direct scavenging action on H_2O_2 and/or hydroxyl radicals. 1,3-Dimethyl-2-thiourea decreased the H_2O_2 concentration in the buffer solution markedly, whereas CP-060S or CP-060R did not modify it, suggesting that neither CP-060S nor CP-060R has a H_2O_2-scavenging effect. In the ESR experiments with DMPO, however, CP-060S and CP-060R decreased the intensity of the DMPO-hydroxyl radical signal in a concentration-dependent way. The second-order rate constant for the reaction of hydroxyl radicals with CP-060S or CP-060R was estimated to be 1.4×10^{10} M^{-1} s^{-1}. This rate constant is higher than that for the reaction of hydroxyl radicals with lecithin (phosphatidylcholine), a major phospholipid in biological membranes, and mannitol, a scavenger of hydroxyl radical. The values for the former and the latter are 5×10^8 and 2.7×10^9 M^{-1} s^{-1}, respectively (Halliwell and Gutterirdge, 1989). Therefore, CP-060S and CP-060R are considered potent scavengers of the hydroxyl radical, which is capable of initiating lipid peroxidation in biological membranes.

Vitamin E and probucol, both of which have a structural component similar to CP-060S [3,5-bis(1,1-dimethylethyl)-4-hydroxyphenyl group], have been shown to attenuate the H_2O_2-induced cytotoxicity in cultured cardiac myocytes (Janero et al., 1991; Horwitz et al., 1996). According to previous findings (Halliwell and Gutterridge, 1989; Gotoh et al. 1992), the phenol-based anti-oxidants, such as vitamin E and probucol, react with lipid peroxyl radicals (which are formed as a consequence of the initiation of lipid peroxidation) rather than hydroxyl radical in biological membranes, and hence prevent propagation of lipid peroxidation. Therefore, the action of CP-060S and CP-060R to reduce the H_2O_2-induced cytotoxicity may be due to scavenging of some radical species including hydroxyl radicals. Further studies are needed to determine the detailed mechanisms of the protective action of CP-060S and CP-060R against the H_2O_2-induced cytotoxicity.

In conclusion, both CP-060S and CP-060R protect the cardiac myocytes from the H_2O_2-induced cytotoxicity through their anti-oxidant activity. CP-060S may be a new type of anti-ischemic agent in terms of having both Ca^{2+} channel blocking and anti-oxidant actions.

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