Pleiotropic Effects of the β-Adrenoceptor Blocker Carvedilol on Calcium Regulation during Oxidative Stress-Induced Apoptosis in Cardiomyocytes

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ABSTRACT

Carvedilol is a nonselective β-adrenoceptor blocker with multiple pleiotropic actions. A recent clinical study suggested that carvedilol may be superior to other β-adrenoceptor blockers in the treatment of heart failure. Despite numerous investigations, the underlying mechanisms of carvedilol on improving heart failure are yet to be fully established. The purpose of this study is to clarify the pleiotropic effect of carvedilol on cytosolic and mitochondrial calcium regulation during oxidative stress-induced apoptosis in cardiomyocytes. Carvedilol (10 μM), but not metoprolol (10 μM), reduced H2O2 (100 μM)-induced apoptosis in neonatal rat cardiomyocytes. During the process, changes in cytosolic calcium concentration ([Ca2+]i) and mitochondrial calcium concentration ([Ca2+]m) were measured by fluorescent probes [Fluo-3/ace-toxymethyl ester (AM), Rhod-2/AM, and tetramethylrhodamine ethyl ester, respectively] and imaged by laser confocal microscopy. The results showed that H2O2 caused [Ca2+]i overload first, followed by [Ca2+]m overload, leading to ΔΨm dissipation and the induction of apoptosis. Carvedilol (10 μM) significantly delayed these processes and reduced apoptosis. These effects were not observed with other β-adrenoceptor blockers (metoprolol, atenolol, and propranolol) or with a combination of the α (phentolamine)- and the β-adrenoceptor blocker. The antioxidant N-acetyl-l-cysteine (NAC, 5 mM) and the combination of NAC and propranolol (10 μM) showed an effect similar to that of carvedilol. Therefore, the effect of carvedilol on H2O2-induced changes in [Ca2+]i, [Ca2+]m, and ΔΨm is independent of α- and β-adrenoceptors but is probably dependent on the antioxidant effect.

Heart failure is now a leading cause of death, and the therapeutic strategy to reduce death from heart failure is an emerging issue in cardiovascular medicine. Despite many previous studies, the underlying mechanisms of heart failure are yet to be established. Oxidative stress has been implicated as an important cause of heart failure (Singal et al., 1998; Giordano, 2005), and the proposed targets of reactive oxygen species are the regulatory system of cytosolic and mitochondrial Ca2+ homeostasis and the mitochondrial respiratory chain (Ide et al., 2001). Recent studies revealed that exposure of cardiomyocytes to H2O2 causes cytosolic and mitochondrial Ca2+ overload and the dissipation of mitochondrial membrane potential (ΔΨm), leading to apoptotic cell death (Korge et al., 2001; Akao et al., 2003). Antioxidants have been shown to inhibit the Ca2+ overload and the mitochondrial dysfunction, resulting in the attenuation of reactive oxygen species-induced cell death (Nakamura et al., 2002; Spallarossa et al., 2004; Giordano, 2005). On the other hand, in the treatment of chronic heart failure, the use of β-adrenoceptor blockers has been established to eliminate excessive β-adrenoceptor stimulation, resulting in the improvement of cardiac function (MERIT-HF Study Group, 1999). However, it remains to be elucidated whether the use...
of β-adrenoceptor blockers attenuates the oxidative stress-induced cytosolic or mitochondrial Ca$^{2+}$ overload in the failing heart.

Carvedilol is a nonselective β-adrenoceptor blocker with multiple pleiotropic actions including an antioxidant and α-adrenoceptor blocking effect. A recent clinical study suggested that carvedilol may be superior to other β-adrenoceptor blockers in the treatment of heart failure (Poole-Wilson et al., 2003). Despite numerous investigations, the underlying mechanisms of carvedilol in improving heart failure are yet to be fully established. The possibility of the underlying mechanisms may be attributable to its antioxidant (Yue et al., 1992), mitochondrial protective (Abreu et al., 2000), or β$_2$-adrenoceptor blocking effects (Molenaar et al., 2006). Therefore, this study investigated whether carvedilol differs from other β-adrenoceptor blockers regarding the regulation of the mitochondrial ([Ca$^{2+}$]$_{mit}$) and cytosolic calcium concentrations ([Ca$^{2+}$]$_{cyt}$) during the process of oxidative stress-induced apoptosis. In addition, the effect of carvedilol on the changes in [Ca$^{2+}$]$_{cyt}$ caused by oxidative stress was compared with that of the antioxidant, N-acetyl-cysteine (NAC).

We show herein that carvedilol prevents dysregulation of [Ca$^{2+}$]$_{mit}$, [Ca$^{2+}$]$_{cyt}$, and ΔΨ$_{m}$ during the process of oxidative stress-induced apoptosis in cardiomyocytes. This effect is unique for carvedilol, independent of the α- and β-adrenoceptor blocking effect and is probably attributable to the antioxidant effect.

Materials and Methods

Materials. Chemicals were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise indicated. Carvedilol (1-[carbazoloyl-4-oxyl]-3-[2-methoxyphenoxoamino]-propanol-2) was provided by Dai-Ichi Pharmaceutical Co. Ltd. (Tokyo, Japan). Carvedilol and atenolol were dissolved in dimethyl sulfoxide. Metoprolol, propranolol, phentolamine, and NAC (Wako Pure Chemicals, Tokyo, Japan) were dissolved in distilled water. The fluorescent dyes were purchased from Molecular Probes (Eugene, OR). All drugs were finally diluted with HEPES buffer (10 mM HEPES, 150 mM NaCl, 5 mM KCl, 1 mM CaCl$_2$, 1 mM MgCl$_2$, and 10 mM glucose, pH 7.4) before use. The final concentration of dimethyl sulfoxide was <0.1%.

Culture of Rat Neonatal Cardiomyocytes. All experiments conformed to the Principles of Laboratory Animal Care (National Institutes of Health publication 85-23, revised 1985) and were approved by the Animal Use and Care Committee of Yamaguchi University Postgraduate School of Medicine. Neonatal cardiomyocytes were isolated from 1-to-2-day-old Wistar rats as described previously (Wang et al., 1998). In brief, ventricles were isolated and incubated at 37°C in a digesting solution containing 0.4% collagenase II (Worthington Biochemicals, Freehold, NJ) and 0.05% trypsin (Difco, Detroit, MI). The cardiomyocytes were purified by Percoll gradient sedimentation and grown in the mixture of Dulbecco’s modified Eagle’s medium and medium 199, supplemented with 10% horse serum, 5% fetal bovine serum, penicillin (100 U/ml), streptomycin (100 μg/ml), and glucose (2 mM). The cardiomyocytes were cultured for 3 to 7 days before the experiment. Twenty-four hours before the experiment, the medium was replaced by a medium supplemented by 0.5% horse serum.

TUNEL Method. Cardiomyocytes were plated on laminin (1 μg/cm$^2$)-precoated four-chamber glass slides and cultured for another 16 h at 37°C with and without H$_2$O$_2$ (50, 100, and 200 μM), and apoptosis was quantified by an apoptotic detection kit (Oncor, Gaithersburg, MD) following the manufacturer’s protocol (Okamura et al., 2000). In brief, the cells were fixed with 10% formalin and treated with proteinase K (20 μg/ml). Endogenous peroxidases were inactivated with 3% H$_2$O$_2$ and treated with terminal deoxynucleotidyl transferase enzyme at 37°C for 90 min. Cells were incubated with the antidigoxigenin peroxidase. Finally, the 3-amino-9-ethylcarbazole peroxidase substrate kit (K0697; Dako North America, Inc., Carpinteria, CA) was used to stain the apoptotic cells. Hematoxylin counterstaining was used to identify the nuclei.

Annexin V-FTIC Binding. To detect the early phase of apoptotic changes, the movement of phosphatidylserine to the extracellular surface was determined by annexin V binding using a commercially available kit (Sigma-Aldrich). The cells were washed twice with phosphate-buffered saline and stained with annexin V-fluorescein isothiocyanate (FITC) and propidium iodide for 15 min at room temperature. Randomly selected microscopic fields (n = 10) were then evaluated to calculate the ratio of fluorescent cells to total cells using an Olympus fluorescent microscope. Images were analyzed using the Metamorph software package (Universal Imaging Corporation, Downingtown, PA).

Cytochrome c Release Measured by Immunoblotting. To measure cytochrome c release from mitochondria to cytosol, which is a critical step in initiating the mitochondria-mediated apoptotic pathway, cardiomyocytes cultured on gelatin-precoated dishes (1.5 × 10$^6$ mm$^2$) were suspended in ice-cold lysis buffer (1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 150 mM NaCl, and 50 mM Tris, pH 7.4) supplemented with protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, 10 μg/ml pepstatin A, 1 mM dithiothreitol, and 5 mM EDTA) just before use. The cells were collected with the cell scraper and centrifuged at 700g for 10 min at 4°C; the supernatant was centrifuged again at 10,000g for 25 min at 4°C. The supernatant was used as a soluble cytoplasmic fraction, which was immunoblotted with an anti-cytochrome c (apoAlert; Clontech, Mountain View, CA) and anti-actin (Oncogene Science, Cambridge, MA). The immune complexes were detected with peroxidase conjugates of anti-rabbit or anti-mouse IgG and then visualized with ECL reagents (Amersham Pharmacia Biotech, Little Chalfont, Buckinghamshire, UK). The contamination of the mitochondrial fraction to cytosolic fraction was tested by immunoblottting for cytochrome c oxidase subunit IV (COX IV; Molecular Probes).

Loading of Fluorescent Dyes. Cardiomyocytes plated on glass dishes were loaded with the following fluorescent dyes: for the measurement of [Ca$^{2+}$]$_{cyt}$, cells were loaded with 3 μM Fluo-3/AM for 20 min at 37°C, and for the measurement of ΔΨ$_{m}$, tetramethylrhodamine ethyl ester (TMRE) was loaded as described previously (Akao et al., 2003). Rhod-2 AM (2 μM) was loaded to measure the [Ca$^{2+}$]$_{mit}$ level at 4°C for 60 min followed by 37°C for 30 min. Mitochondrial tracker green (MGT) (50 nM) was loaded for 20 min at the room temperature.

Confocal Fluorescence Measurements and Image Analysis. Cardiomyocytes were illuminated, and images were acquired with a model 510 laser confocal scanning microscope (Carl Zeiss Inc., Thornwood, NY). To determine [Ca$^{2+}$]$_{cyt}$, the cells were excited by the 488 nm emission line of an argon laser, and Fluo-3 fluorescence was collected between 505 and 530 nm. For measurement of ΔΨ$_{m}$ and [Ca$^{2+}$]$_{mit}$, cells were excited at 543 nm from a helium-neon laser, and the fluorescence of TMRE and Rhod-2 was detected at 605 ± 16 nm. Confocal microscopic images were taken every 5 min using a ×40 objective lens. Regions of interest were created surrounding the individual cells, and the temporal changes of individual cells were averaged for 10 cells.

Statistical Analysis. The data are presented as means ± S.D. Multiple comparisons among the groups were carried out using one-way analysis of variance with Fisher’s least significant difference as the post hoc test. A value of p < 0.05 was considered to be statistically significant.

Results

Effect of Carvedilol and Metoprolol on H$_2$O$_2$-Induced Apoptosis. Without H$_2$O$_2$, only a few TUNEL-posi-
tive nuclei were observed after 16 h of cardiomyocyte culture. Carvedilol (10 μM) or metoprolol (10 μM) per se did not affect apoptosis. The number of TUNEL-positive nuclei increased dose-dependently with H₂O₂ (Fig. 1A), and carvedilol (10 μM) significantly decreased H₂O₂-induced TUNEL-positive cells, but metoprolol (10 μM) did not show such an effect. Another estimate of apoptosis, the number of annexin V-FITC-positive cardiomyocytes, was investigated after exposure to H₂O₂ with and without carvedilol (10 μM) or metoprolol (10 μM) (Fig. 1B). Carvedilol decreased significantly the number of annexin V-FITC positive cardiomyocytes. In contrast, metoprolol did not show such an effect.

H₂O₂-Induced Cytochrome c Release. The release of cytochrome c from the mitochondria is a crucial step in the induction of apoptosis (Skulachev, 1998). After the cardiomyocytes were exposed to H₂O₂ for 40 min, a significant amount of cytochrome c release was detected by immunoblot (Fig. 1C). Carvedilol (10 μM) significantly suppressed the cytochrome c release to 41 ± 13% (P < 0.01), but metoprolol (10 μM) did not (78 ± 11%, N.S.) (Fig. 1D).

Effect of Carvedilol on [Ca²⁺]ᵢ. More than 85% of the neonatal cardiomyocytes showed spontaneous beating at a frequency of >40 beats/min. Therefore, the images of the Fluo-3 fluorescence obtained by confocal laser microscopy reflected [Ca²⁺]ᵢ, including both systolic and diastolic phases. In the present study, the diastolic phase of fluorescence intensity was measured and normalized to the initial value. Without H₂O₂, the Fluo-3 fluorescence of the cardiomyocytes remained unchanged for at least 60 min (Fig. 2A). Once H₂O₂ was added, Fluo-3 fluorescence started to increase (Fig. 2B). When cardiomyocytes were preincubated with carvedilol (1, 5, and 10 μM), the increase of Fluo-3 fluorescence induced by H₂O₂ was attenuated (Fig. 2, C, D, and E, respectively). The dose-dependent effect of carvedilol on the time course of Fluo-3 fluorescence after exposure to H₂O₂ is illustrated in Fig. 2F and the statistical analysis at 20, 30, and 40 min after exposure to H₂O₂ is shown in Fig. 2G. The Fluo-3 fluorescence was attenuated significantly by 1 μM carvedilol at 20 and 30 min after H₂O₂ stimulation. The IC₅₀ value was calculated as 4.8 μM from the dose-response curve of carvedilol at 40 min after H₂O₂ stimulation (Fig. 2H). The effect of carvedilol was compared with that of other β-adrenoceptor blockers (Fig. 2I). However, neither metoprolol (10 μM) nor propranolol (10 μM) had influence on Fluo-3 fluorescence.

Fig. 1. Effect of carvedilol and metoprolol on H₂O₂-induced apoptosis. A, cardiomyocytes were exposed to H₂O₂ for 16 h with and without carvedilol (Carv, 10 μM) or metoprolol (Met, 10 μM). Apoptosis was quantified using the TUNEL method. B, effect of carvedilol and metoprolol on H₂O₂ (100 μM)-induced apoptosis detected by the annexin V-FITC binding assay. C, representative immunoblot of cytosolic cytochrome c (Cyt-c) separated by SDS-polyacrylamide gel electrophoresis, transferred onto a nitrocellulose membrane. Actin is used as an internal control. D, densitometric analysis of cytochrome c release. H₂O₂ (100 μM)-induced cytochrome c release from mitochondria to cytosolic fraction. Results are mean ± S.D. of three experiments. *, p < 0.05 and **, p < 0.01 versus H₂O₂ without drugs.
Fig. 2. Effect of carvedilol on $[\text{Ca}^{2+}]_i$. A to E, representative sequential images of Fluo-3 fluorescence with and without $	ext{H}_2\text{O}_2$. Cells without $	ext{H}_2\text{O}_2$ maintained a low constant level of $[\text{Ca}^{2+}]_i$ (A). Cardiomyocytes were exposed to $	ext{H}_2\text{O}_2$ (B), and the effect of carvedilol at 1 $\mu$M (C), 5 $\mu$M (D), and 10 $\mu$M (E) on Fluo-3 fluorescence is shown. Bar, 50 $\mu$m. F, effects of various concentrations of carvedilol on the temporal changes of Fluo-3 fluorescence. G, effect of carvedilol on Fluo-3 fluorescence at 20, 30, and 40 min after the exposure to $	ext{H}_2\text{O}_2$. H, a dose-response curve showing Fluo-3 fluorescence to carvedilol at 40 min after exposure to $	ext{H}_2\text{O}_2$. The $\text{IC}_{50}$ was calculated as 4.8 $\mu$M. I, effect of carvedilol and other drugs on temporal changes of Fluo-3 fluorescence after exposure to $	ext{H}_2\text{O}_2$. Fluo-3 fluorescence is expressed as a percentage of initial levels. Results are means ± S.D. of three experiments. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ versus without carvedilol.
Fig. 3. Effect of carvedilol on H$_2$O$_2$-induced ΔΨ$_m$. A to E, representative sequential images of TMRE fluorescence. Control cells maintained a constant fluorescence level (A). Cardiomyocytes were exposed to H$_2$O$_2$ (B) and coincubated with carvedilol 1 μM (C), 5 μM (D), and 10 μM (E). Bar, 50 μm. F, effect of various concentrations of carvedilol on the temporal changes of TMRE fluorescence expressed as a percentage of initial levels after exposure to H$_2$O$_2$. G, effect of carvedilol on TMRE fluorescence at 40, 50, and 60 min after exposure to H$_2$O$_2$. H, the dose-response curve of carvedilol. IC$_{50}$ was 4.8 μM. I, effect of β-adrenoceptor blockers other than carvedilol and/or the α-adrenoceptor blocker on the temporal changes of TMRE fluorescence after exposure to H$_2$O$_2$. TMRE fluorescence is expressed as a percentage of the initial value. Results are means ± S.D. of four experiments. *, p < 0.05; **, p < 0.01 versus without carvedilol.
Effect of Carvedilol on $\Delta \Psi_m$. The TMRE fluorescence reflecting $\Delta \Psi_m$ remained unchanged in the untreated control cardiomyocytes (Fig. 3A). TMRE fluorescence started to decrease approximately 40 min after $\text{H}_2\text{O}_2$ application (Fig. 3B). Carvedilol (1, 5, and 10 $\mu$M) inhibited the dissipation of TMRE fluorescence induced by $\text{H}_2\text{O}_2$ (Fig. 3, C, D, and E, respectively). The time course of TMRE fluorescence changes is illustrated (Fig. 3F). The changes in TMRE fluorescence were analyzed at 40, 50, and 60 min after the $\text{H}_2\text{O}_2$ exposure (Fig. 3G). The IC$_{50}$ value was calculated as 4.8 $\mu$M from the dose-response curve of carvedilol at 60 min after $\text{H}_2\text{O}_2$ stimulation (Fig. 3H). The effect of carvedilol was compared with that of other $\beta$-adrenoceptor blockers (Fig. 3I). However, neither metoprolol (10 $\mu$M) nor propranolol (10 $\mu$M) had influence on the time course of TMRE fluorescence induced by $\text{H}_2\text{O}_2$.

To clarify the cause-effect relation between intracellular calcium overload and $\Delta \Psi_m$, 1,2-bis(2-aminophenoxy)ethane-$N,N,N',N'$-tetraacetic acid-acetoxymethyl ester (BAPTA-AM) (25 $\mu$M), an intracellular Ca$^{2+}$ chelator, was added to the cells under $\text{H}_2\text{O}_2$ exposure. The depolarization of $\Delta \Psi_m$ was completely eliminated (Fig. 4). This finding indicates that intracellular Ca$^{2+}$ overload is essential for the impairment of mitochondrial function by oxidative stress.

Comparison of Carvedilol and Other Drugs on $[\text{Ca}^{2+}]_i$ and $\Delta \Psi_m$. The latencies of the $[\text{Ca}^{2+}]_i$ increase and $\Delta \Psi_m$ dissipation after exposure to $\text{H}_2\text{O}_2$ are summarized in Fig. 5. Carvedilol (10 $\mu$M) significantly prolonged the latency period for the $[\text{Ca}^{2+}]_i$ rise from 21.3 $\pm$ 0.6 to 42.5 $\pm$ 4.7 min ($p < 0.01$) (Fig. 5A). The latency period of the $\Delta \Psi_m$ dissipation was 40.8 $\pm$ 3.3 min, which was significantly longer than that of the $[\text{Ca}^{2+}]_i$ rise. Carvedilol (10 $\mu$M) significantly prolonged the latency of the $\Delta \Psi_m$ dissipation to 86.5 $\pm$ 9.7 min ($p < 0.001$) (Fig. 5A). The difference of the latency between $[\text{Ca}^{2+}]_i$ rise and $\Delta \Psi_m$ dissipation was dose-dependently prolonged by carvedilol (Fig. 5B), suggesting a protective effect of carvedilol on mitochondria.
From mitochondria. In the process of the H$_2$O$_2$-induced apoptosis, we showed that carvedilol ameliorated and delayed [Ca$^{2+}$]$_i$ and [Ca$^{2+}$]$_m$ overload, which subsequently delayed ΔΨ$_m$ dissipation. These findings were not observed with other β$_1$-selective adrenoceptor blockers metoprolol or atenolol. Furthermore, a nonselective β-adrenoceptor blocker, propranolol, and the combination of propranolol and an α-adrenoceptor blocker, phenolamine, also failed to exert the same effect as that observed with carvedilol.

Regarding the mechanism of H$_2$O$_2$-induced apoptosis in cardiomyocytes, calcium dysregulation in mitochondria has been shown to be critically important because calcium overload in mitochondria causes a respiratory chain abnormality, which subsequently causes ΔΨ$_m$ dissipation through the opening of permeability transition pores (Akao et al., 2003; Teshima et al., 2003; Long et al., 2004). Thus, we determined the temporal sequences of the changes in [Ca$^{2+}$]$_i$, and [Ca$^{2+}$]$_m$ by H$_2$O$_2$. The rise in [Ca$^{2+}$]$_m$ by H$_2$O$_2$ was demonstrated to be an earlier event than the [Ca$^{2+}$]$_i$ rise, leading to the dissipation of ΔΨ$_m$. These findings are in accord with those of a recent study showing that the diastolic level of mitochondrial Ca$^{2+}$ was elevated earlier than the diastolic level of cytosolic Ca$^{2+}$ from the calcium transient analysis (Robert et al., 2001). The mechanism may be explained by the fact that the efflux of Ca$^{2+}$ from the mitochondria is slower than that from the cytosol, leading to an earlier accumulation of Ca$^{2+}$ in the mitochondria than in the cytosol under the oxidative stress (Robert et al., 2001). However, contradictory results have been shown that the increase in [Ca$^{2+}$]$_i$, preceded the onset of [Ca$^{2+}$]$_m$ rise (Teshima et al., 2003). The reason for the discrepancy is unknown.

This study further verified the cause-effect relationship between the increase in [Ca$^{2+}$]$_i$, and the dissipation of ΔΨ$_m$ by H$_2$O$_2$. The intracellular Ca$^{2+}$ chelator BAPTA-AM completely inhibited the dissipation of ΔΨ$_m$ by H$_2$O$_2$, indicating that the [Ca$^{2+}$]$_m$ overload is the trigger of the ΔΨ$_m$ dissipation. However, the result should be carefully interpreted because the Ca$^{2+}$ chelator stops beating of the cardiomyocytes, probably leading to inhibition of the dissipation of ΔΨ$_m$.

The cardioprotective effect of carvedilol has been shown in a variety of in vitro and in vivo models (Yue et al., 1998; Spallarossa et al., 2004). However, the underlying mechanisms are yet to be determined because carvedilol is known to be a β-blocker with multiple pleiotropic effects, a β-adrenergic blocking effect, and antioxidant and proteasome action on the mitochondria (Oliveira et al., 2000). One most likely mechanism of cardioprotection by carvedilol is the antioxidant effect (Nakamura et al., 2002; de Groot et al., 2004). However, it remained unclear whether the effect of carvedilol on [Ca$^{2+}$]$_i$, under H$_2$O$_2$ is attributable to its antioxidant effect. We therefore examined the effect of the antioxidant NAC on it. NAC at 5 mM inhibited the [Ca$^{2+}$]$_i$ overload under H$_2$O$_2$, but the combination of propranolol with NAC failed to promote the protective effect of NAC. Therefore, it is suggested that the effect of carvedilol is mainly dependent on its antioxidant effect, and a synergistic effect of the β-adrenoceptor blocking effect is unlikely.

The antioxidant effect of carvedilol has been shown to be much more potent than other β-adrenoceptor blockers when examined in rat brain homogenates (Yue et al., 1992). The IC$_{50}$ value of carvedilol was reported to be 8.1 μM and that

**Discussion**

The present study has demonstrated that the β-adrenoceptor blocker with multiple pleiotropic effects, carvedilol, reduced H$_2$O$_2$-induced apoptotic cell death, which was confirmed by three different methods including TUNEL staining, annexin V-FITC staining, and cytochrome c release from mitochondria. In the process of the H$_2$O$_2$-induced apoptosis, we showed that carvedilol ameliorated and delayed [Ca$^{2+}$]$_i$ and [Ca$^{2+}$]$_m$ overload, which subsequently delayed ΔΨ$_m$ dissipation. These findings were not observed with other β$_1$-selective adrenoceptor blockers metoprolol or atenolol. Furthermore, a nonselective β-adrenoceptor blocker, propranolol, and the combination of propranolol and an α-adrenoceptor blocker, phenolamine, also failed to exert the same effect as that observed with carvedilol.

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This study further verified the cause-effect relationship between the increase in [Ca$^{2+}$]$_i$, and the dissipation of ΔΨ$_m$ by H$_2$O$_2$. The intracellular Ca$^{2+}$ chelator BAPTA-AM completely inhibited the dissipation of ΔΨ$_m$ by H$_2$O$_2$, indicating that the [Ca$^{2+}$]$_m$ overload is the trigger of the ΔΨ$_m$ dissipation. However, the result should be carefully interpreted because the Ca$^{2+}$ chelator stops beating of the cardiomyocytes, probably leading to inhibition of the dissipation of ΔΨ$_m$.

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of atenolol to be 1.0 mM. A similar result was also shown in isolated liver mitochondria (Abreu et al., 2000). In the present study, we proved that the antioxidant effect is unique only for carvedilol and not for other β-adrenoceptor blockers, such as metoprolol, propranolol, and atenolol. The IC<sub>50</sub> value of carvedilol in cardiomyocytes is 4.8 μM with [Ca<sup>2+</sup>]<sub>i</sub> and ΔΨ<sub>mi</sub>. This result is in accordance with the findings of previous studies (Yue et al., 1992; Flesch et al., 1999; de Groot et al., 2004; Koitabashi et al., 2005).

Because cardiomyocytes beat spontaneously during the experiments, one important issue to be considered is whether the changes in beating rate and the contractility influence [Ca<sup>2+</sup>]<sub>i</sub>. The intracellular calcium changes are characterized by systolic and the diastolic components. The systolic component may be influenced by the changes in beating rate and contractility. However, the diastolic component is much less influenced by the factors. In the present study, we measured the diastolic component of the intracellular calcium as [Ca<sup>2+</sup>]<sub>i</sub>. Therefore, the changes in beating rate and contractility caused by the β-adrenoceptor blockers and/or H<sub>2</sub>O<sub>2</sub> should not influence the result of the present study.

We conclude that carvedilol ameliorates the [Ca<sup>2+</sup>]<sub>m</sub> and [Ca<sup>2+</sup>]<sub>i</sub> overload induced by H<sub>2</sub>O<sub>2</sub>, leading to a delay in ΔΨ<sub>mi</sub> dissipation and inhibition of apoptosis. This action of carvedilol is independent of its α- and β-adrenoceptor blocking effects and probably attributable to its antioxidant effect.

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