

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

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Abbreviations: ROS, reactive oxygen species; $\Delta\Psi_m$, mitochondrial membrane potential; $[Ca^{2+}]_m$, mitochondrial calcium concentration; $[Ca^{2+}]_i$, cytosolic calcium concentration; NAC, N-acetyl-L-cysteine; TUNEL, TdT-mediated dUTP nick-end labeling; TMRE, tetramethylrhodamine ethyl ester; MTG, mitochondrial tracker green; BAPTA-AM, 1,2-bis-(2-aminophenoxy)ethane-*N,N,N',N'*-tetraacetic acid-acetoxy-methyl ester; PTP, permeability transition pore; ANOVA, analysis of variance.

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Abstract

Carvedilol is a non-selective 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 μ M), but not metoprolol (10 μ M), reduced H_2O_2 (100 μ M) -induced apoptosis in neonatal rat cardiomyocytes. During the process, changes in cytosolic calcium concentration ($[\text{Ca}^{2+}]_i$) and mitochondrial calcium concentration ($[\text{Ca}^{2+}]_m$) and mitochondrial membrane potential ($\Delta\Psi_m$) were measured by fluorescent probes (Fluo-3 AM, rhod-2 AM and TMRE, respectively) and imaged by laser confocal microscopy. The results showed that H_2O_2 caused $[\text{Ca}^{2+}]_m$ overload first, then followed by the $[\text{Ca}^{2+}]_i$ overload, leading to the $\Delta\Psi_m$ dissipation and the induction of apoptosis. Carvedilol (10 μ M) significantly delayed these processes and reduced apoptosis. These effects were not observed in other β -adrenoceptor blockers (metoprolol, atenolol, propranolol) or 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 μ M) showed the similar effect to carvedilol. Therefore, the effect of carvedilol on H_2O_2 -induced changes in $[\text{Ca}^{2+}]_m$, $[\text{Ca}^{2+}]_i$ and $\Delta\Psi_m$ is independent of α - and β -adrenoceptors, but likely dependent on the antioxidant effect.

Introduction

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 (Giordano, 2005; Singal et al., 1998) and the proposed targets of reactive oxygen species (ROS) are the regulatory system of cytosolic and mitochondrial Ca^{2+} homeostasis, and the mitochondrial respiratory chain (Ide T, et al., 2001). Recent studies revealed that H_2O_2 exposure to cardiomyocytes causes cytosolic and mitochondrial Ca^{2+} overload, and the dissipation of mitochondrial membrane potential ($\Delta\Psi_m$), leading to apoptotic cell death (Korge et al., 2001; Akao et al., 2003). Antioxidants have been shown to inhibit the Ca^{2+} overload and the mitochondrial dysfunction, resulting in the attenuation of ROS-induced cell death (Giordano, 2005; Nakamura et al., 2002; Spallarossa et al., 2004). 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 non-selective β -adrenoceptor blocker with multiple pleiotropic actions including the 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 on 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), a mitochondrial protective (Abreu et al., 2000), or the 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 ($[Ca^{2+}]_m$) and cytosolic calcium concentrations ($[Ca^{2+}]_i$) during the process of oxidative stress-induced apoptosis. Additionally, the effect of carvedilol on the changes in $[Ca^{2+}]_i$ caused by oxidative stress was compared to that of the antioxidant, N-acetyl-L-cysteine (NAC).

We herein show that carvedilol prevents dysregulation of $[Ca^{2+}]_i$, $[Ca^{2+}]_m$, and $\Delta\Psi_m$ 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 likely 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)-oxy]-3-[(2-methoxyphenoxyethyl)-amino]-propanol-2} was provided from Dai-Ichi Pharmaceutical Co. Ltd. (Tokyo, Japan). Carvedilol and atenolol were dissolved in dimethylsulfoxide (DMSO). Metoprolol, propranolol, phentolamine and N-acetyl-L-cysteine (NAC, Wako, Japan) were dissolved in distilled water. The fluorescent dyes were purchased from Molecular Probes (Eugene, OR). All drugs were finally diluted with 2-[4-(2-Hydroxyethyl)-1-piperazinyl]-ethanesulfonic acid (HEPES) buffer (10 mM HEPES, 150 mM NaCl, 5 mM KCl, 1 mM CaCl₂, 1 mM MgCl₂, 10 mM glucose, pH 7.4.) before use. The final concentration of DMSO was less than 0.1%.

Culture of rat neonatal cardiomyocyte

All experiments conformed to the *Guide for the Care and Use of Laboratory Animals* (NIH publication) and were approved by the Animal Use and Care Committee of Yamaguchi University Post-graduate School of Medicine. Neonatal cardiomyocytes were isolated from 1-to-2-day-old Wistar rats as described previously (Wang et al., 1997). Briefly, ventricles were isolated and incubated at 37°C in a digesting solution containing 0.4% collagenase II (Worthington Biochemical) and 0.05% trypsin (DIFCO). The cardiomyocytes were purified by the 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 μ g/ml), and glutamine (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.

TdT-mediated dUTP nick-end labeling (TUNEL) method

Cardiomyocytes were plated on laminin (1 μ g/cm²) -precoated 4-chamber-glass slides and cultured for another 16 h at 37°C with and without H₂O₂ (50, 100 and 200 μ M) and apoptosis was quantified by apoptotic detection kit (Oncor, USA) following the manufacture's protocol (Okamura et al., 2000). Briefly, the cells were fixed with 10% formalin, treated with proteinase K (20 μ g/ml). Endogenous peroxidases were inactivated with 3% H₂O₂ and treated with TdT enzyme at 37°C for 90 min. Cells were incubated with the anti-digoxigenin peroxidase. Finally, the 3-amino-9-ethylcarbazole peroxidase substrate kit (K0697, Dako) was used to stain the apoptotic cells. The hematoxylin counterstaining was used to identify the nuclei.

AnnexinV- fluorescein isothiocyanate (FITC) 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). The cells were washed twice with PBS and stained with annexin V-FITC and propidium iodide (PI) for 15 minutes 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).

Cytochrome c release measured by immunoblotting

To measure the 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 /60 mm) were suspended in ice-cold lysis buffer (1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 150 mM NaCl, and 50 mM Tris, pH 7.4) supplemented with protease inhibitors (1 mM phenylmethylsulfonylfluoride, 1 μ g/ml leupeptin, 10 μ g/ml pepstatin A, 1 mM dithiothreitol, and 5 mM ethylenediaminetetracetic acid) just before use. The cells were collected with the cell scraper and centrifuged at 700 g for 10 min at 4°C, the supernatant was centrifuged again at 10,000 g for 25 min at 4°C. The supernatant was used as a soluble cytoplasmic fraction, which was immunoblotted with anti-cytochrome *c* (Clontech apoAlert) and anti-actin (Oncogene Sciences). The immune complexes were detected with peroxidase conjugates of anti-rabbit or anti-mouse IgG and then visualized with ECL reagents (Amersham Pharmacia Biotech, Inc). The contamination of the mitochondrial fraction to cytosolic fraction was tested by immunoblotting for cytochrome c oxidase subunit IV (COX IV, Molecular Probes, Eugene, OR).

Loading of fluorescent dyes

Cardiomyocytes plated on glass dishes were loaded with the following fluorescent dyes; for the measurement of $[Ca^{2+}]_i$, cells were loaded with 3 μ M Fluo-3 AM for 20 min at 37°C and for the measurement of $\Delta\Psi_m$, tetramethylrhodamine ethyl ester (TMRE) was loaded as described previously (Akao et al., 2003). Rhod-2 AM (2 μ M) was loaded to measure $[Ca^{2+}]_m$ level at 4°C for 60 min followed by 37°C for 30 min. Mitochondrial tracker green (MTG, 50 nM) was loaded for 20 min at the room temperature.

Confocal fluorescence measurements and the image analysis

Cardiomyocytes were illuminated and images were acquired with a Model 510 laser confocal scanning microscope (Zeiss). To determine the $[Ca^{2+}]_i$, 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 measuring $\Delta\Psi_m$ and $[Ca^{2+}]_m$, 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 \times 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 the means \pm SD. Multiple comparisons among the groups were carried out using one-way ANOVA 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 H₂O₂-induced apoptosis

Without H₂O₂, only a few TUNEL-positive nuclei were observed after 16 h of cardiomyocyte culture. Carvedilol (10 μM) or metoprolol (10 μM) per se did not affect the number of apoptosis. The number of TUNEL-positive nuclei increased dose-dependently by H₂O₂ (Fig. 1A), and carvedilol (10 μM) significantly decreased the H₂O₂-induced TUNEL positive cells, but metoprolol (10 μM) did not show such 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 %, ns) (Fig.1D).

Effect of carvedilol on [Ca²⁺]_i

More than 85% of the neonatal cardiomyocytes showed spontaneous beating at a frequency of over 40 bpm. Therefore, the images of the Fluo-3 fluorescence obtained

by confocal laser microscopy reflected the $[Ca^{2+}]_i$ 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 H_2O_2 , the Fluo-3 fluorescence of the cardiomyocytes remained unchanged for at least 60 min (Figures 2A). Once H_2O_2 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 H_2O_2 was attenuated (Fig. 2C, D, and E, respectively). The dose-dependent effect of carvedilol on the time course of Fluo-3 fluorescence after exposure to H_2O_2 was illustrated in Fig. 2F and the statistical analysis at 20, 30 and 40 min after exposure to H_2O_2 was shown in Fig. 2G. The Fluo-3 fluorescence was attenuated significantly by 1 μM carvedilol at 20 and 30 min after H_2O_2 stimulation. The IC_{50} value was calculated as 4.8 μM from the dose-response curve of carvedilol at 40 min after the H_2O_2 stimulation (Fig 2H). The effect of carvedilol was compared with other β -adrenoceptor blockers (Fig. 2I). However, neither metoprolol (10 μM) nor propranolol (10 μM) had influence on Fluo-3 fluorescence.

Effect of carvedilol on $\Delta\Psi_m$

The TMRE fluorescence reflecting the $\Delta\Psi_m$ remained unchanged in the untreated control cardiomyocytes (Figures 3A). TMRE fluorescence started to decrease approximately 40 minutes after the H_2O_2 application (Figures 3B). Carvedilol (1, 5, and 10 μM) inhibited the dissipation of TMRE fluorescence induced by H_2O_2 (Fig. 3C, D, and E, respectively). The time course of TMRE fluorescence changes is illustrated

(Figure 3F). The changes in TMRE fluorescence were analyzed at 40, 50 and 60 min after the H₂O₂ exposure (Fig 3G). The IC₅₀ value was calculated as 4.8 μM from the dose-response curve of carvedilol at 60 min after the H₂O₂ stimulation (Fig 3H). The effect of carvedilol was compared to other β-adrenoceptor blockers (Fig. 3I). However, neither metoprolol (10 μM) nor propranolol (10 μM) had influence on the time-course of TMRE fluorescence induced by H₂O₂.

To clarify the cause-effect relation between intracellular calcium overload and ΔΨ_m, 1,2-bis(2-aminophenoxy)ethane-*N,N,N,N'*-tetraacetic acid-acetoxymethyl ester (BAPTA-AM, 25 μM), an intracellular Ca²⁺ chelator, was added to the cells under H₂O₂ exposure. The depolarization of ΔΨ_m was completely eliminated. This finding indicates that intracellular Ca²⁺ overload is essential for the impairment of mitochondrial function by oxidative stress.

Comparison of carvedilol and other drugs on [Ca²⁺]_i and ΔΨ_m

The latency of [Ca²⁺]_i increase and ΔΨ_m dissipation after the exposure to H₂O₂ was summarized in Fig.5. Carvedilol (10 μM) significantly prolonged the latency period for the [Ca²⁺]_i rise from 21.3 ± 0.6 min to 42.5 ± 4.7 min (*p*<0.01) (Fig 5A). The latency period of the ΔΨ_m dissipation was 40.8 ± 3.3 min which was significantly longer than that of the [Ca²⁺]_i rise. Carvedilol (10 μM) significantly prolonged the latency of the ΔΨ_m dissipation to 86.5 ± 9.7 min (*p*<0.001) (Fig 5A). Difference of the latency between [Ca²⁺]_i rise and ΔΨ_m dissipation was dose-dependently prolonged by carvedilol (Fig 5B), suggesting a protective effect of carvedilol on mitochondria.

On the other hand, neither selective β_1 -adrenoceptor blockades (metoprolol, atenolol) nor non-selective β -adrenoceptor blockade (propranolol) had effects on the latency of $[Ca^{2+}]_i$ rise (Fig 5C) and $\Delta\Psi_m$ dissipation (Fig 5D). Since the IC_{50} values for isoproterenol inhibition of beta-blockers examined in the present study is as low as nM order, we thus chose the concentration of 1 and 10 μ M which should be enough to completely block the β -adrenergic receptors.

Because carvedilol has an α -adrenoceptor blocking effect, the effect of an α -adrenoceptor blocker (phentolamine, 10 μ M), and the combination of propranolol (10 μ M) and phentolamine (10 μ M) on the H_2O_2 -induced $[Ca^{2+}]_i$ and $\Delta\Psi_m$ changes were examined and neither had any influence. However, NAC (5 mM) did prolong the latency of $[Ca^{2+}]_i$ rise, but no additional effect of propranolol (10 μ M) was observed.

Effect of carvedilol on H_2O_2 -induced $[Ca^{2+}]_m$

Rhod-2 and MTG were co-loaded to the cardiomyocytes. The fluorescence of rhod-2 reflecting the $[Ca^{2+}]_m$ increased after the H_2O_2 stimulation (Fig. 6A). The latency of the $[Ca^{2+}]_m$ rise was prolonged by carvedilol (10 μ M) from 15.7 ± 0.58 min to 21.3 ± 0.6 min ($p < 0.01$). The same effect was not observed by metoprolol (10 μ M).

Discussion

The present study has demonstrated that the β -adrenoceptor blocker with multiple pleiotropic effects, carvedilol, reduced the H_2O_2 -induced apoptotic cell death, which was confirmed by three different methods including TUNEL staining, annexin V-FITC staining, and the cytochrome *c* release from mitochondria. In the process of the H_2O_2 -induced apoptosis, we showed that carvedilol ameliorated and delayed the $[\text{Ca}^{2+}]_i$ and $[\text{Ca}^{2+}]_m$ overload, which subsequently delayed the $\Delta\Psi_m$ dissipation. These findings were not observed by other β_1 -selective adrenoceptor blockades, metoprolol or atenolol. Furthermore, a non-selective β -adrenoceptor blockade, propranolol, and the combination of propranolol and an α -adrenoceptor blockade, phentolamine, also failed to exert the same effect as that observed in carvedilol.

Regarding the mechanism of H_2O_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 $\Delta\Psi_m$ dissipation through the opening of permeability transition pores (Akao et al., 2003; Long et al., 2004; Teshima et al., 2003). Thus, we determined the temporal sequences of the changes in $[\text{Ca}^{2+}]_m$ and $[\text{Ca}^{2+}]_i$ by H_2O_2 . The rise in $[\text{Ca}^{2+}]_m$ by H_2O_2 was demonstrated to be an earlier event than the $[\text{Ca}^{2+}]_i$ rise, leading to the dissipation of $\Delta\Psi_m$. These findings accord with the 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 $[\text{Ca}^{2+}]_i$ preceded the onset of $[\text{Ca}^{2+}]_m$ rise (Teshima et al., 2003). The reason for the discrepancy is unknown.

This study further verified the cause–effect relationship between the increase in $[\text{Ca}^{2+}]_i$ and the dissipation of $\Delta\Psi_m$ by H_2O_2 . Intracellular Ca^{2+} chelator, BAPTA-AM, completely inhibited the dissipation of $\Delta\Psi_m$ by H_2O_2 , indicating that the $[\text{Ca}^{2+}]_i$ overload is the trigger of the $\Delta\Psi_m$ dissipation. However, the result should be carefully interpreted because Ca^{2+} chelator stops beating of the cardiomyocytes, likely leading to inhibiting the dissipation of $\Delta\Psi_m$.

The cardioprotective effect of carvedilol has been shown in a variety of in vitro and in vivo models (Spallarossa et al., 2004; Yue et al., 1998). However, the underlying mechanisms are yet to be determined because carvedilol is known to be a β -blocker with multiple pleiotropic effects; α -adrenergic blocking effect, antioxidant and protonophoretic action on the mitochondria (Oliveira et al., 2000). One most likely mechanism of cardioprotection by carvedilol is the antioxidant effect (Groot et al., 2004; Nakamura et al., 2002). However, it remained unclear whether the effect of carvedilol on $[\text{Ca}^{2+}]_i$ under H_2O_2 is attributable to its antioxidant effect. We therefore examined the effect of antioxidant, NAC, on it. The 5 mM NAC inhibited the $[\text{Ca}^{2+}]_i$ overload under H_2O_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 the 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 blockades when examined in the rat brain homogenate (Yun et al., 1992). The IC_{50} value of carvedilol was reported to be 8.1 μ M, and that of atenolol to be 1.0 mM. A similar result was also shown in isolated liver mitochondria (Abrea et al., 2000). In the present study, we proved that the antioxidant effect is unique only for carvedilol, not for other β -adrenoceptor blockers, such as metoprolol, propranolol and atenolol. The IC_{50} value of carvedilol in cardiomyocytes is 4.8 μ M regarding the $[Ca^{2+}]_i$ and $\Delta\Psi_m$. This result is in accordance with the findings of previous studies (Flesh et al., 1999; de Groot et al., 2004; Koitabashi et al., 2005; Yun et al., 1992).

Since cardiomyocytes beat spontaneously during the experiments, one important issue to be considered is whether the changes in beating rate and the contractility influence on $[Ca^{2+}]_i$. The intracellular calcium changes are characterized by the 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 $[Ca^{2+}]_i$. Therefore, the changes in beating rate and contractility caused by the β -adrenoceptor blockers and/or H_2O_2 should not influence the result of the present study.

We conclude that carvedilol ameliorates the $[Ca^{2+}]_m$ and $[Ca^{2+}]_i$ overload by H_2O_2 , leading to the delay of $\Delta\Psi_m$ dissipation and inhibiting apoptosis. This action of

carvedilol is independent of the α - and β -adrenoceptor blocking effect and likely attributable to its antioxidant effect.

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References

- Abreu RM, Santos DJ and Moreno AJ (2000) Effects of carvedilol and its analog BM-910228 on mitochondrial function and oxidative stress. *J Pharmacol Exp Ther* 295:1022-1030.
- Akao M, Rourke BO, Teshima Y, Seharaseyon J and Marban E (2003) Mechanistically distinct steps in the mitochondrial death pathway triggered by oxidative stress in cardiac myocytes. *Circ Res* 92:186-194.
- Flesch M, Maack C, Cremers B, Baumer AT, Sudkamp M and Bohm M (1999) Effect of β -blockers on free radical-induced cardiac contractile dysfunction. *Circulation* 100:346-353.
- Giordano, F.J (2005) Oxygen, oxidative stress, hypoxia, and heart failure. *J Clin Invest* 115:500-508.
- de Groot AA, Mathy MJ, van Zwieten PA and Peters SL (2004) Antioxidant activity of nebivolol in the rat aorta. *J Cardiovasc Pharmacol* 43:148-153.
- Ide T, Tsutsui H, Hayashidani S, Kang D, Suematsu N, Nakamura K, Utsumi H, Hamasaki N, and Takeshita A (2001) Mitochondrial DNA damage and dysfunction associated with oxidative stress in failing hearts after myocardial infarction. *Circ Res* 88:529–535.
- Koitabashi N, Arai M, Tomaru K, Takizawa T, Watanabe A, Niwano K, Yokoyama T, Wuytack F, Periasamy M, Nagai R and Kurabayashi M (2005) Carvedilol effectively blocks oxidative stress-mediated downregulation of sarcoplasmic reticulum Ca^{2+} -ATPase 2 gene transcription through modification of Sp1 binding.

Biochem Biophys Res Commun 328:116-124.

Korge P, Honda HM and Weiss JN (2001) Regulation of the mitochondrial permeability transition by matrix Ca^{2+} and voltage during anoxia/reoxygenation.

Am J Physiol Cell Physiol 280:C517-526.

Long X, Goldenthal MJ, Wu GM and Marin-Garcia J (2004) Mitochondrial Ca^{2+} flux and respiratory enzyme activity decline are early events in cardiomyocyte response to H_2O_2 . *J Mol Cell Cardiol* 37:63-70.

Molenaar P, Christ T, Ravens U and Kaumann A (2006) Carvedilol blocks β_2 - more than β_1 -adrenoceptors in human heart. *Cardiovasc Res* 69:128-139.

MERIT-HF Study Group. (1999) Effect of metoprolol CR=XL in chronic heart failure: Metoprolol CR=XL randomised intervention trial in congestive heart failure (MERIT-HF). *Lancet* 353:2001-2007.

Nakamura K, Kusano K, Nakamura Y, Kakishita M, Ohta K, Nagase S, Yamamoto M, Miyaji K, Saito H, Morita H, Emori T, Matsubara H, Toyokuni S and Ohe T (2002) Carvedilol decreases elevated oxidative stress in human failing myocardium. *Circulation* 105:2867-2871.

Okamura T, Miura T, Takemura G, Fujiwara H, Iwamoto H, Kawamura S, Kimura M, Ikeda Y, Iwatate M and Matsuzaki M (2000) Effect of caspase inhibitors on myocardial infarct size and myocyte DNA fragmentation in the ischemia-reperfused rat heart. *Cardiovasc Res* 45:642-650.

Oliveira PJ, Marques MP, Batista de Carvalho LA, Moreno AJ (2000) Effects of carvedilol on isolated heart mitochondria: evidence for a protonophoretic

mechanism. *Biochem Biophys Res Commun* 276:82-87.

Poole-Wilson PA, Swedberg K, Cleland JG, Di Lenarda A, Hanrath P, Komajda M, Lubsen J, Lutiger B, Metra M, Remme WJ, Torp-Pedersen C, Scherhag A, Skene A and Carvedilol Or Metoprolol European Trial Investigators (2003) Comparison of carvedilol and metoprolol on clinical outcomes in patients with chronic heart failure in the Carvedilol Or Metoprolol European Trial (COMET): randomised controlled trial. *Lancet* 362:7-13.

Robert V, Gurlini P, Tosello V, Nagai T, Miyawaki A, Di Lisa F and Pozzan T (2001) Beat-to-beat oscillations of mitochondrial $[Ca^{2+}]$ in cardiac cells. *EMBO J* 20:4998-5007.

Singal PK, Khaper N, Palace V and Kumar D (1998) The role of oxidative stress in the genesis of heart disease. *Cardiovasc Res* 40:426-432.

Skulachev VP (1998) Cytochrome c in the apoptotic and antioxidant cascades. *FEBS Lett* 423:275-280.

Spallarossa P, Garibaldi S, Altieri P, Fabbi P, Manca V, Nasti S, Rossettin P, Ghigliotti G, Ballestrero A, Patrone F, Barsotti A and Brunelli C (2004) Carvedilol prevents doxorubicin-induced free radical release and apoptosis in cardiomyocytes in vitro. *J Mol Cell Cardiol* 37:837-846.

Teshima Y, Akao M, Jones SP and Marban E (2003) Cariporide (HOE642), a selective Na^+-H^+ exchange inhibitor, inhibits the mitochondrial death pathway. *Circulation* 108:2275-2281.

Wang Y, Huang S, Sah VP, Ross J Jr, Brown JH, Han J, Chien KR (1998) Cardiac

muscle cell hypertrophy and apoptosis induced by distinct members of the p38 mitogen-activated protein kinase family. *J Biol Chem* 273:2161-2168.

Yue TL, Cheng HY, Lysko PG, McKenna PJ, Feuerstein R, Gu JL, Lysko KA, Davis LL, Feuerstein G (1992) Carvedilol, a new vasodilator and β -Adrenoceptor antagonist, is an antioxidant and free radical scavenger. *J Pharmacol Exp Ther* 263:92-98.

Yue TL, Ma XL, Wang X, Romanic AM, Liu GL, Loudon C, Gu JL, Kumar S, Poste G, Ruffolo RR Jr and Feuerstein GZ (1998) Possible involvement of stress-activated protein kinase signaling pathway and Fas receptor expression in prevention of ischemia/reperfusion-induced cardiomyocyte apoptosis by carvedilol. *Circ Res* 82:166-174.

Footnotes

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Legends for Figures

Fig. 1. Effect of carvedilol and metoprolol on H₂O₂-induced apoptosis. A, cardiomyocytes were exposed to H₂O₂ for 16 h with and without carvedilol (10 μM) or metoprolol (10 μM). Apoptosis was quantified using TUNEL method. B, effect of carvedilol and metoprolol on H₂O₂ (100 μM)-induced apoptosis detected by annexin V-FITC binding assay. C, representative immunoblot of cytosolic cytochrome *c* separated by SDS-PAGE, transferred onto 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. Car, carvedilol 10 μM; Met, metoprolol 10 μM. 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 [Ca²⁺]_i. A-E, representative sequential images of Fluo-3 fluorescence with and without H₂O₂. Cells without H₂O₂ maintained a low constant level of [Ca²⁺]_i (A). Cardiomyocytes were exposed to H₂O₂ (B), and the effect of carvedilol 1 μM (C), 5 μM (D), and 10 μM (E) on Fluo-3 fluorescence were shown. The bar indicates 50 μm. F, effects of various concentrations of carvedilol on the temporal changes of Fluo-3 fluorescence. G, effect of carvedilol on TMRE fluorescence at 40, 50 and 60 min after the exposure to H₂O₂. H, a dose-response curve showing TMRE fluorescence to carvedilol at 40 min after exposure to H₂O₂. IC₅₀ was calculated as 4.8 μM. I, effect of carvedilol and other drugs on temporal changes of Fluo-3 fluorescence after exposure to H₂O₂. Fluo-3 fluorescence is

expressed as a percentage of initial levels. Results are mean \pm S.D. of three experiments. *, $p < 0.05$; **, $p < 0.01$ and *** $p < 0.001$ versus without carvedilol.

Fig. 3. Effect of carvedilol on H₂O₂-induced $\Delta\Psi_m$. A-E, representative sequential images of TMRE fluorescence. Control cells maintained a constant fluorescence level (A). Cardiomyocytes were exposed to H₂O₂ (B), and co-incubated with carvedilol 1 μ M (C), 5 μ M (D), and 10 μ M (E). The bar indicates 50 μ m. F, effect of various concentrations of carvedilol on the temporal changes of TMRE fluorescence expressed as a percentage of initial levels after exposure to H₂O₂. G, effect of carvedilol on TMRE fluorescence at 40, 50 and 60 min after exposure to H₂O₂. H, dose-response curve of carvedilol. IC₅₀ was 4.8 μ M. I, effect of other β -adrenoceptor blockers than carvedilol and/or the α -adrenoceptor blocker on the temporal changes of TMRE fluorescence after exposed to H₂O₂. TMRE fluorescence is expressed as a percentage of initial value. Results are mean \pm S.D. of four experiments. *, $p < 0.05$; **, $p < 0.01$ versus without carvedilol.

Fig. 4. Effect of the intracellular calcium chelator, BAPTA-AM (25 μ M), on the TMRE fluorescence expressed as a percentage of initial levels after stimulated by H₂O₂. With BAPTA-AM, the dissipation of $\Delta\Psi_m$ was completely suppressed.

Fig. 5. Effect of carvedilol on the latency of $\Delta\Psi_m$ loss and the [Ca²⁺]_i increase after the exposure to H₂O₂. A, the latency for $\Delta\Psi_m$ is longer than that for [Ca²⁺]_i. Carvedilol

prolonged the latency for both $\Delta\Psi_m$ and $[Ca^{2+}]_i$ dose-dependently. * $p < 0.05$; ** $p < 0.01$ and *** $p < 0.001$ vs. without carvedilol regarding $\Delta\Psi_m$, †† $p < 0.01$ versus without carvedilol regarding $[Ca^{2+}]_i$. B, the difference of latencies between $\Delta\Psi_m$ loss and $[Ca^{2+}]_i$ increase. * $p < 0.05$; ** $p < 0.01$ vs. without carvedilol. Data are mean \pm S.D. from four experiments. C and D, effects of carvedilol, and other drugs on the latency for $\Delta\Psi_m$ loss and $[Ca^{2+}]_i$ increase. Carv, carvedilol; Met, metoprolol; Prop, propranolol; Aten, atenolol; Phen, phentolamine; Prop+Phen, the combination of propranolol(10 μ M) and phentolamine (10 μ M); NAC, N-acetyl-L-cysteine (5 mM); Prop+NAC, the combination of propranolol(10 μ M) and NAC (5 mM); Vehi, vehicle. White bar indicates 1 μ M and black bar 10 μ M. ** $p < 0.01$ and *** $p < 0.001$ versus Vehi. Data are mean \pm S.D. from four experiments.

Fig. 6. Effect of carvedilol and metoprolol on $[Ca^{2+}]_m$. A, representative images of cardiomyocytes loaded with Rhod-2 AM (red) and MTG (Green). The bar indicates 50 μ m. After the exposure to H_2O_2 , Rhod-2 fluorescence increased indicating calcium overload in the mitochondria. B, effects of carvedilol (10 μ M) and metoprolol (10 μ M) on the latency of $[Ca^{2+}]_m$ rise. * $p < 0.05$ versus control. Results are mean \pm S.D. of three experiments.

Figure 1

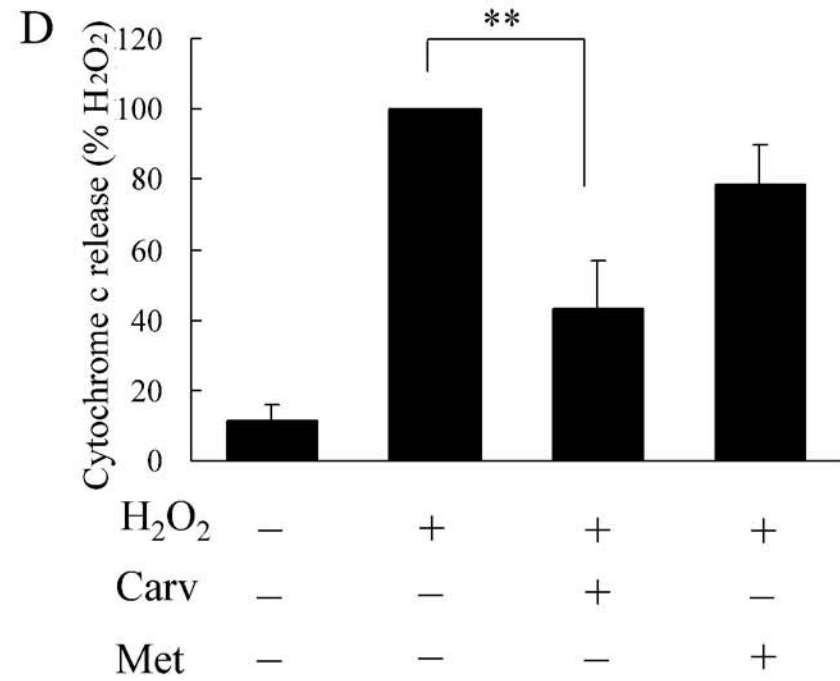
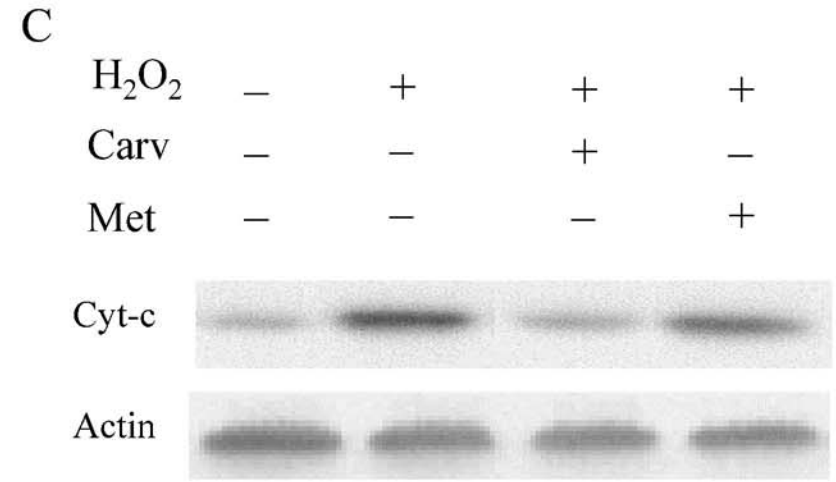
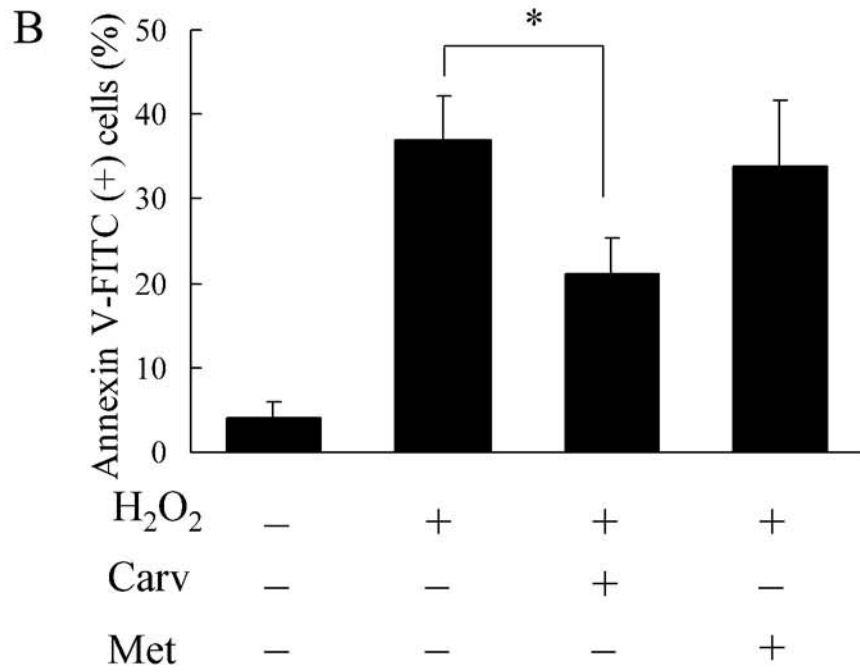
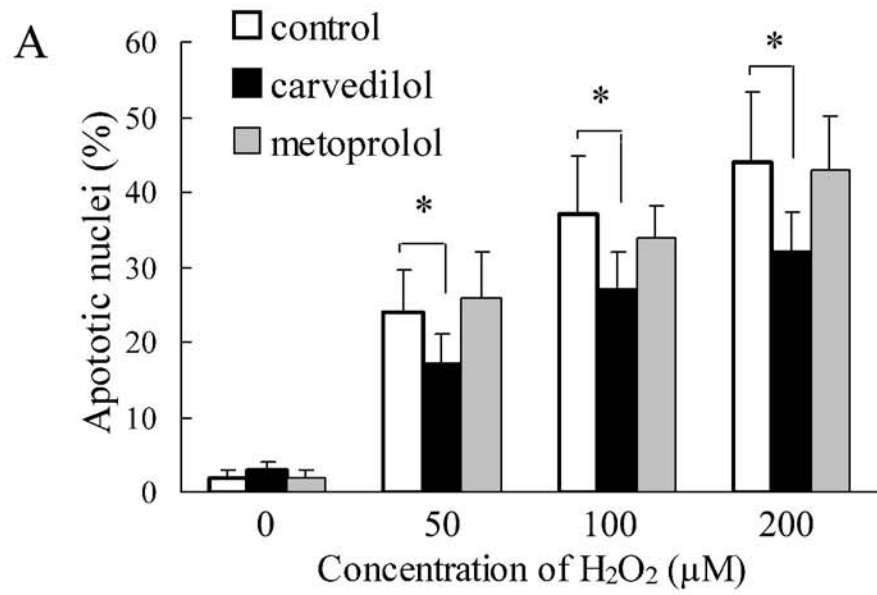
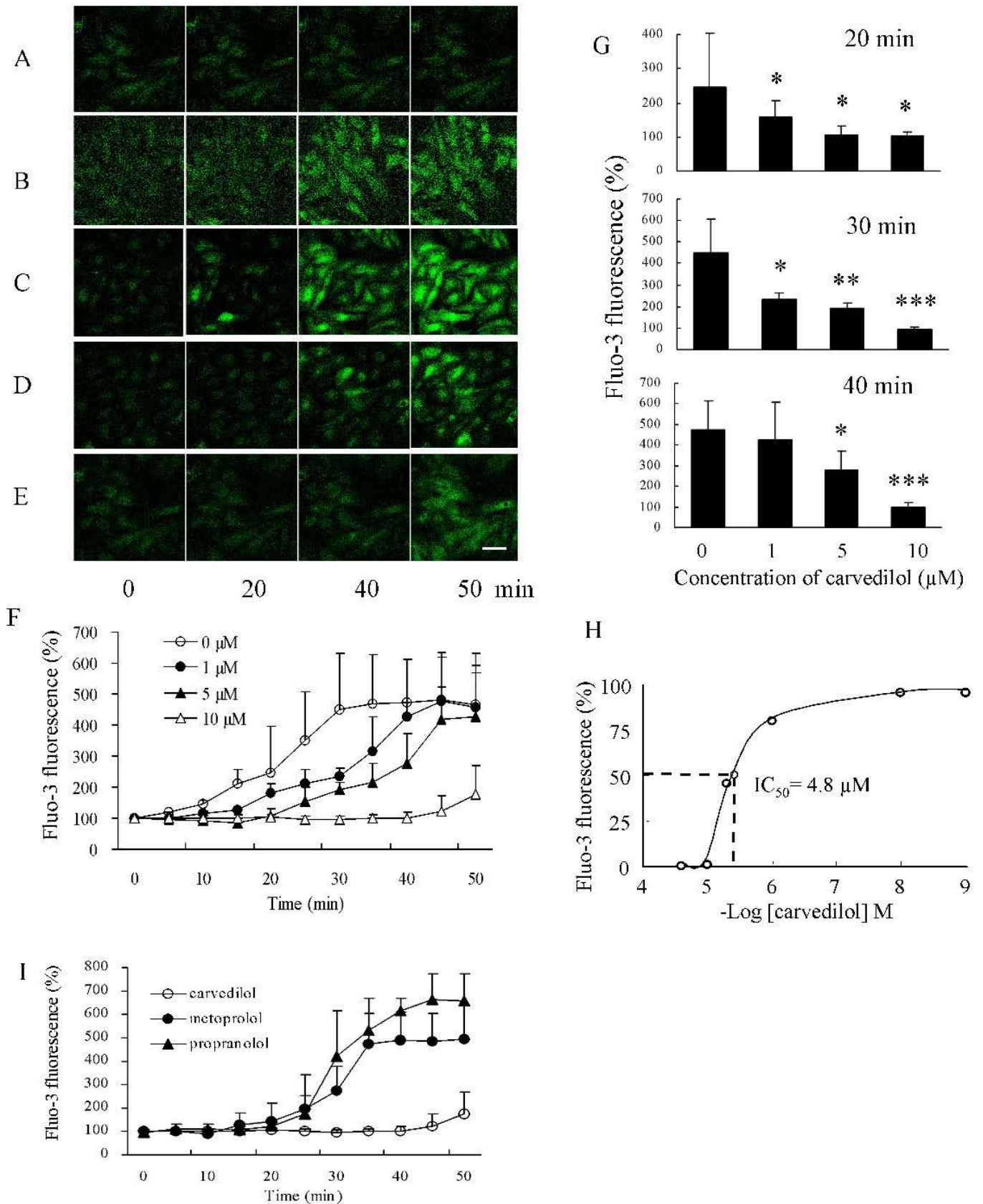


Figure 2



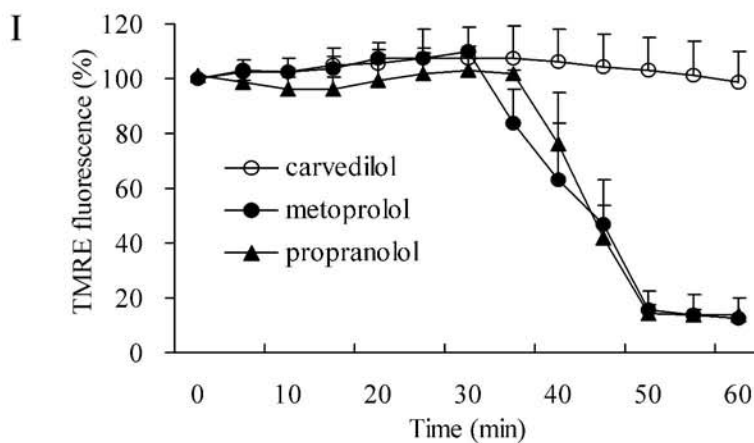
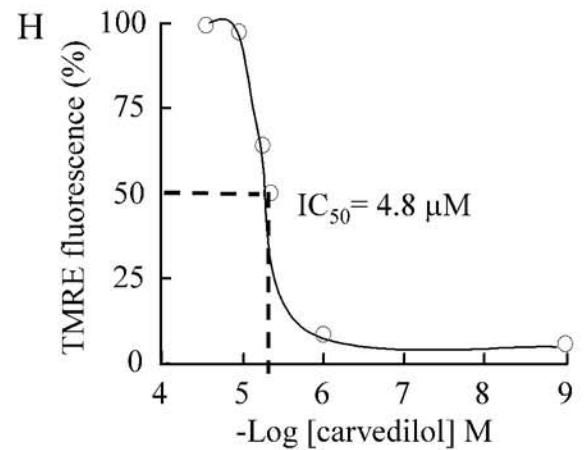
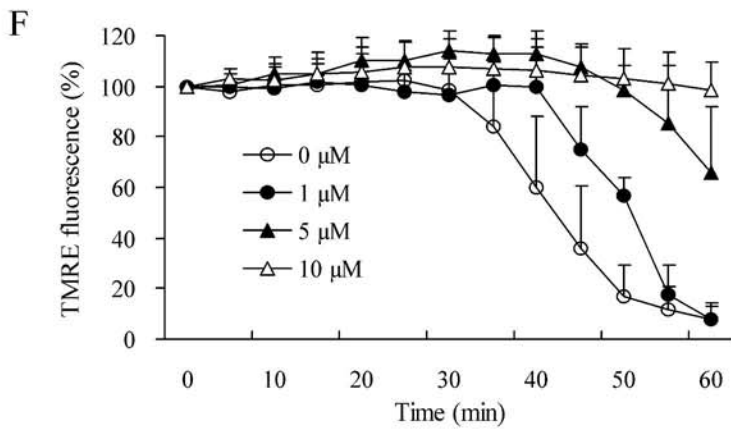
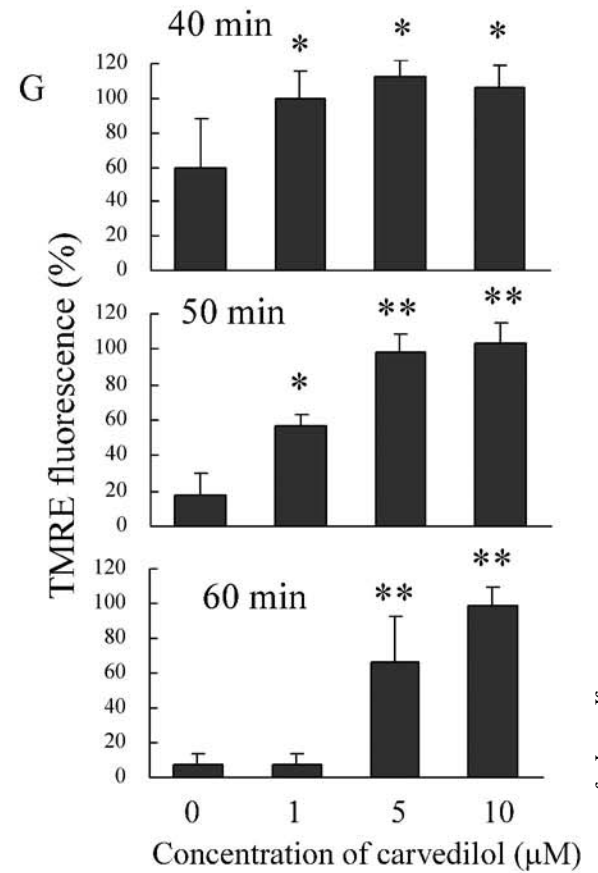
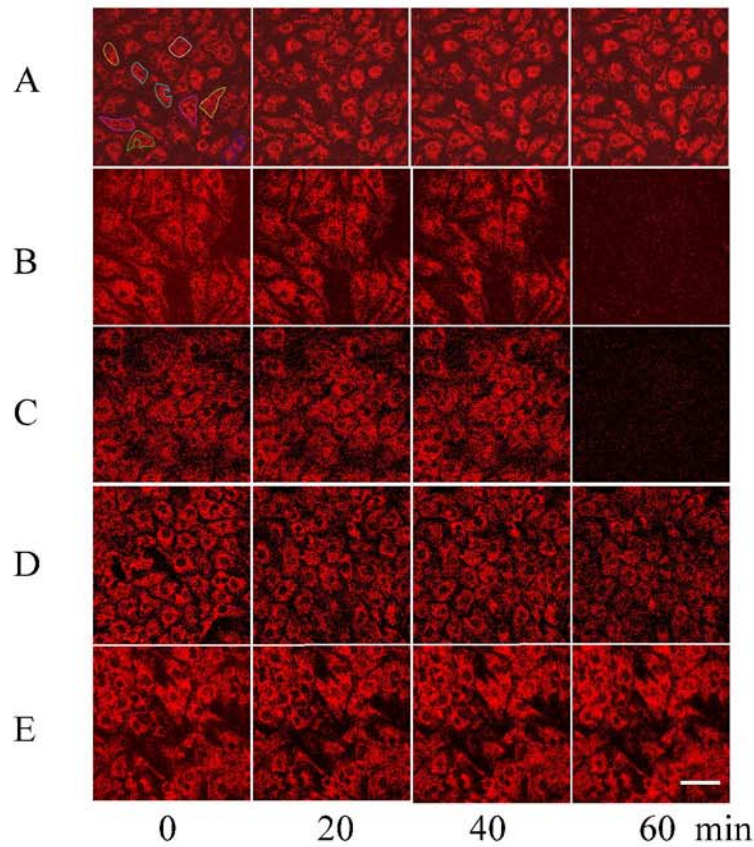


Figure 4

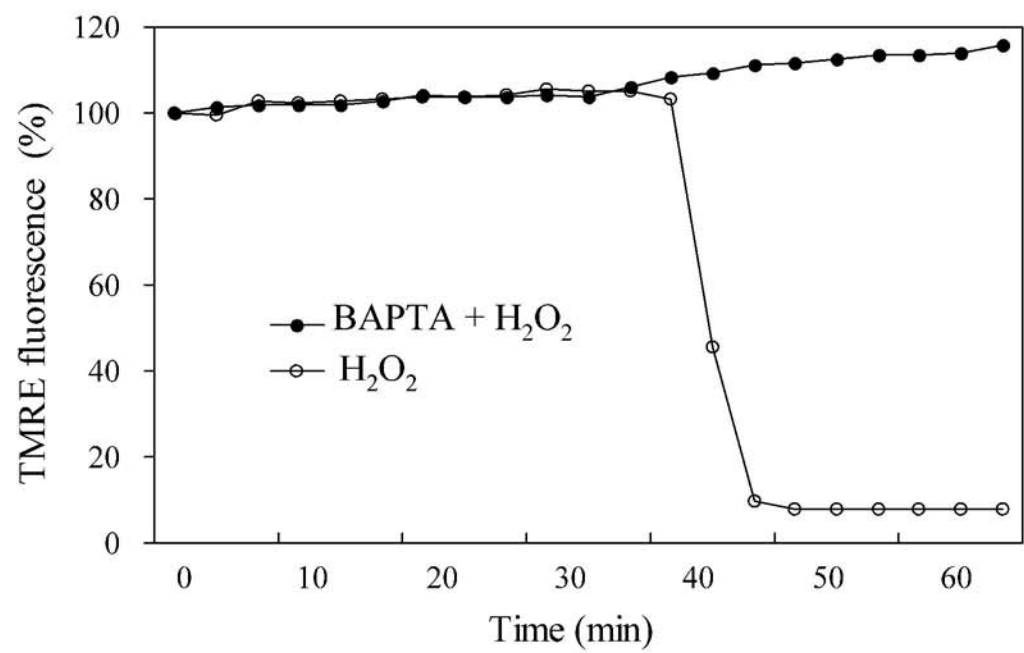


Figure 5

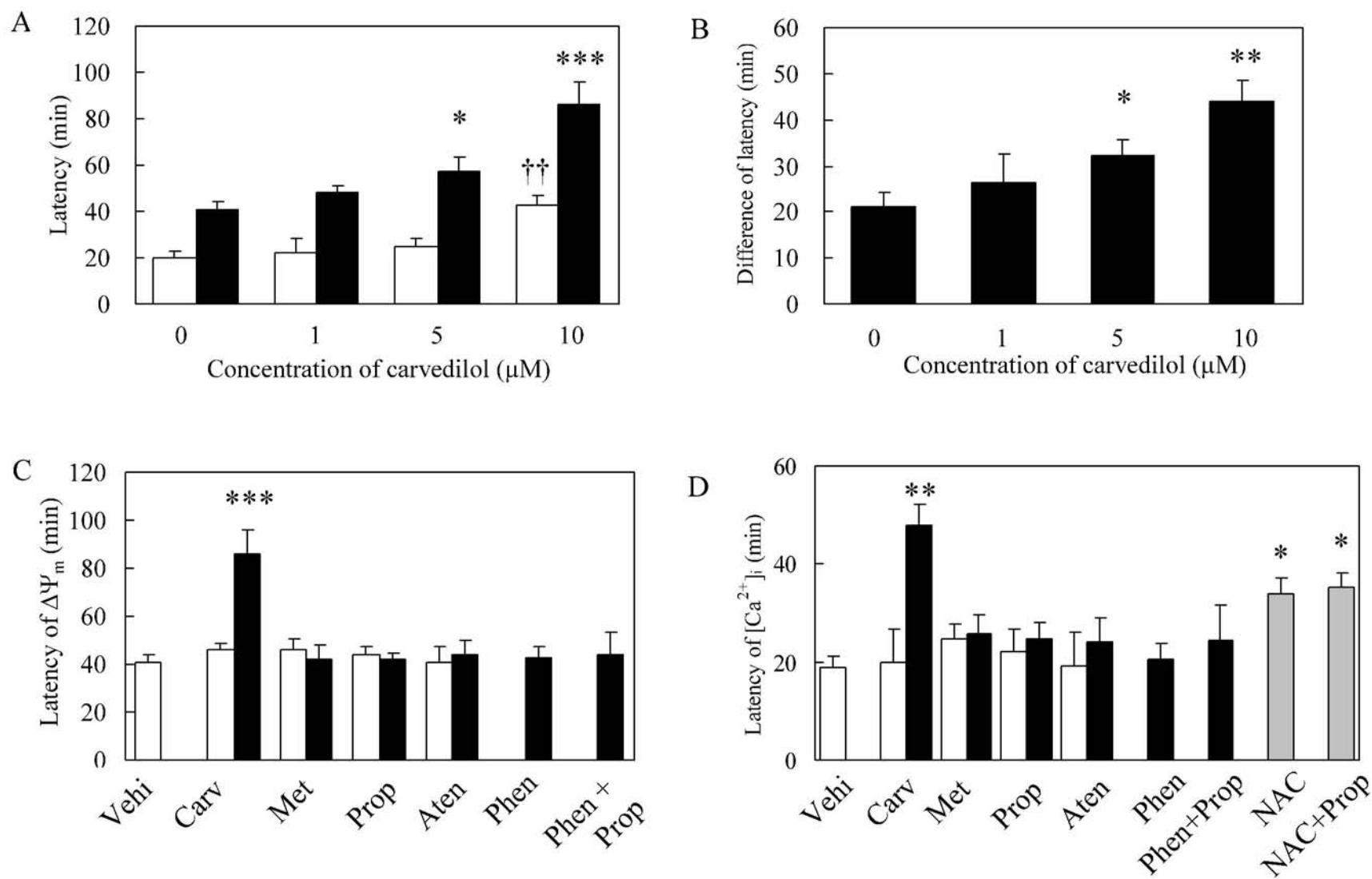


Figure 6

