Background: Long-term administration of nitroglycerin (NTG) causes tolerance secondary to increased vascular formation of reactive oxygen species. Carvedilol, which has potent antioxidant activity in addition to functioning as an adrenergic blocker, prevents nitrate tolerance by a still to be elucidated mechanism. The present study investigated how carvedilol attenuates nitrate tolerance, particularly with reference to cytochrome P450 (CYP), an enzyme involved in the development of tolerance.

Methods and Results: Male Wistar rats were subjected to 48-h continuous infusion of NTG alone (0.5mg/h) or NTG with concomitant carvedilol (20 or 100μg/h), and then compared with vehicle-treated rats (4 groups; n=6 in each group). Following the continuous administration, nitrate tolerance, assessed by bolus NTG injections, was hemodynamically prevented by coadministration of carvedilol. Levels of CYP1A1/1A2, superoxide production, and phosphorylated vasodilator-stimulated phosphoprotein at serine 239 (P-VASP) were examined in the aortic wall and heart tissue. When NTG alone was continuously administered, vascular superoxide was produced, there was a decrease in the cardiac CYP1A1/1A2 level, and depletion of P-VASP. However, each of these changes induced by continuous NTG administration was significantly attenuated by coadministration of carvedilol and the extent of attenuation was more pronounced at the higher dose (100μg/h).

Conclusions: Coadministration of carvedilol attenuates nitrate tolerance through maintenance of NO/cGMP pathway activity by preventing free radical generation and CYP depletion. (Circ J 2010; 74: 1711–1717)

Key Words: Carvedilol; Cytochrome P450; Nitrate tolerance; Oxidative stress

Carvedilol is a combined β1-, β2- and α1-adrenergic blocking agent approved for the treatment of hypertension, chronic heart failure, and left ventricular dysfunction following myocardial infarction.1–5 A large body of experimental and clinical evidence has demonstrated that carvedilol is advantageous in comparison with other β-blockers,5–7 because of its potent antioxidant activity.6–10 Oxidative stress has generally been considered of cardinal importance in the pathogenesis of cardiovascular diseases,11 and antioxidant supplementation has received attention because of the possibility that it prevents or can be used to treat cardiovascular diseases.12 Thus, the antioxidative effect of carvedilol appears likely to be of considerable importance in the field of cardiovascular disease.6,9,10

Nitroglycerin (NTG) is another standard cardiovascular agent and is still frequently used for treating acute/chronic angina and congestive heart failure. When given acutely, its effectiveness is undisputable. However, its efficacy with long-term administration is limited because of the loss of hemodynamic and anti-ischemic effects, a phenomenon termed “nitrate tolerance”.7,13–21 The etiology of nitrate tolerance remains incompletely understood and is certainly multifactorial.17–20 According to previous investigations, its primary cause is increased oxidative stress,17–20 with key enzymes possibly including mitochondrial aldehyde dehydrogenase-2 (ALDH-2)20,23 and cytochrome P450 (CYP)18,21–23 Munzel et al noted that ALDH-2 was responsible for bioactivation of NTG applied in low concentrations, and that CYP was involved in the metabolism of high concentrations of NTG.23 We have reported several observations of the contribution of CYP to the development and attenuation of nitrate tolerance.18,21

Carvedilol has already been demonstrated to prevent the development of nitrate tolerance in randomized clinical studies,7,16 though the mechanism responsible has not been clearly determined. This study was therefore designed to elu-
cidate how carvedilol attenuates the development of nitrate tolerance, particularly the involvement of the CYP system.

**Methods**

**Chemicals**

NTG was given as a 0.5 mg/ml solution (Mirisrol; Nihon Kayaku Co, Tokyo, Japan). Carvedilol (Daichisankyo Co, Ltd, Tokyo, Japan) was dissolved in dimethyl sulfoxide and stored as a 10-mmol/L stock solution. Anti-phosphorylated vasodilator-stimulated phosphoprotein (P-VASP) (Ser239) and anti-VASP antibodies were purchased from Calbiochem (San Diego, CA, USA).

### Animal Model

Male Wistar rats (8 weeks of age, 220–240 g; SLC, Shizuoka, Japan) were anesthetized with 50 mg/kg pentobarbital for a catheterization procedure as described previously. A catheter (polyethylene tubing) filled with heparin (100 IU/ml) was placed in the femoral vein and externalized through the dorsal skin along the subdermal space, so that rats could freely move in a cage while agents were continuously administered through the femoral line. After catheter implantation, rats were allowed to recover until they appeared healthy and were then housed individually with free access to standard rat chow and tap water under a 12 h light-dark cycle.

Rats were divided into 4 groups according to administration of the following agents (6 rats per group). NTG (0.5 mg/ml) or vehicle (saline) was continuously infused at 1 ml/h for 48 h using a syringe pump (Model CFV-3200; Nihon Kohdent, Tokyo, Japan), as described previously (NTG and Vehicle groups, respectively). Carvedilol at a dose of 20 or 100 μg/h was concomitantly administered for 48 h with continuous NTG infusion at the same dose as in the NTG group (Carv20 and Carv100 groups, respectively). At the end of 48-h continuous administration of the respective agents, rats were cannulated through the opposite femoral artery and vein under urethane anesthesia (1 g/kg, ip). The femoral arterial line was connected to a pressure transducer (Baxter, Tokyo, Japan), and blood pressure was recorded on a polygraph (Model RM-6200; Nihon Koden, Tokyo, Japan). To assess the development of nitrate tolerance, additional NTG (0.01, 0.03, 0.1, 0.3, 1, or 3 mg/kg) was injected through the femoral vein. Other rats were similarly treated and then killed just after the 48-h continuous administration of the respective agents. The aorta and heart were removed immediately and stored in liquid nitrogen until analyses. Some pieces of these tissues were frozen in optimal cutting temperature (OCT) compound for in situ O2•− analysis. This study conformed to the Guide for the Care and Use of Laboratory Animals approved by the authorities of the local ethics review board on experimental animal research, which is based on the US National Institutes of Health guidelines.

**Immunoblot Analysis**

The aortic wall and heart tissues were each homogenized and sonicated in 0.4 ml of 50 mmol/L Tris-HCl, pH 7.5, containing 1 mmol/L EDTA, 150 mmol/L NaCl, 10% glycerol, 1 mmol/L NaVO4, 0.2 mmol/L N-tosyl-L-phenylalanyln chloromethyl ketone, 0.1 mmol/L N-a-tosyl-L-lysyl chloromethyl ketone, and a protease inhibitor cocktail tablet (Roche Diagnostics GmbH, Mannheim, Germany). Homogenates were centrifuged at 12,000 g for 10 min. The supernatants from the aortic wall (10 μg) were examined by immunoblot analysis for P-VASP and total VASP using 7.5% gels as described next. Anti-P-VASP was used for the analysis of P-VASP, a reliable biochemical marker of vascular cyclic guanosine monophosphate-activated protein kinase I activity. Cytosolic fractions from the heart tissue were centrifuged at 105,000 g for 60 min at 4°C. The resulting microsomal fractions were separated on 7.5% gels by SDS-PAGE and transferred electrophoretically to PVDF membranes.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Vehicle</th>
<th>NTG (0.5 mg/h)</th>
<th>Carv20</th>
<th>Carv100</th>
</tr>
</thead>
<tbody>
<tr>
<td>NTG (0.5 mg/h)</td>
<td>−</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Carvedilol (μg/h)</td>
<td>−</td>
<td>−</td>
<td>20</td>
<td>100</td>
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</table>
Because CYP1A1/1A2 isoforms have already been demonstrated to be the most potent mediators of nitric oxide (NO) formation from NTG in rats,21 blots were probed with a specific antibody to CYP1A1/1A2 (Chemicon, Temecula, CA, USA). All the immunoreactive bands were visualized by enhanced chemiluminescence (Amersham International, Buckinghamshire, UK). All antibodies were diluted with Can Get Signal (Toyobo, Osaka, Japan) following the manufacturer’s instructions. Western blots were quantified using Scion image software (NIH Image version 1.63). The intensities of bands were determined as relative intensities to the mean value of control samples.

**Determination of Intracellular Generation of Superoxides**

Aortic superoxide production was determined as described previously.18 Briefly, the oxidative fluorescent dye, dihydroethidium (DHE: Wako Chemical, Osaka, Japan), was used to evaluate the in situ production of superoxides. Unfixed fresh aortic wall and heart tissues frozen in OCT compound were cut into 10-μm sections and placed on glass slides. DHE (40 μmol/L) was topically applied to each tissue section and the slides were incubated in a light-protected humidified chamber at 37°C for 30 min as described previously.18 Images were obtained with a confocal laser scanning microscope (Carl Zeiss: LSM-510) equipped with a krypton/argon laser. Laser settings were identical for the acquisition of images from all specimens. Fluorescence was detected with a 585-nm long-pass filter. Some specimens with superoxide dismutase (100 U/ml) exhibited no intensity.

**Statistical Analysis**

Values are presented as means±SEM, with differences considered significant at P<0.05. Statistical examination was performed by analysis of variance (ANOVA), with differences in decrease in blood pressure among the groups examined by 2-way-ANOVA. Statistical analyses were performed using the StatView 5.0 software package (SAS Institute, Cary, NC, USA).

![Figure 2](image2.png) **Figure 2.** Decreases in blood pressure following various bolus doses of nitroglycerin (NTG) injection (0.01–0.3 mg/kg) in each group, as shown in Figure 1. (●) NTG group; (▲) Carv20 group, (◆) Carv100 group, (○) Vehicle group. All datapoints in the NTG group differed significantly from those in the Vehicle group (P<0.01). *P<0.05 vs NTG group, †P<0.05 between the Carv20 and Carv100 groups.

![Figure 3](image3.png) **Figure 3.** Effects of continuous coadministration of carvedilol on in situ detection of superoxides in aortic wall and heart sections. Rats were treated as shown in Figure 1. Fluorescent photomicrographs show confocal microscopic sections of heart samples labeled with the oxidative dye dihydroethidium (exhibiting red fluorescence when oxidized to ethidium bromide by O2•−). Representative scans for the Vehicle, nitroglycerin (NTG), and Carv20 groups are shown from the left to right, respectively (A, aortic wall; B, heart sections). (Original magnification, ×200; scale bar=40 μm.)
Results

Effects of Continuously Coadministered Carvedilol on Bolus NTG-Induced Decrease in Blood Pressure

Representative results of a single bolus NTG injection (0.3 mg/kg) are shown in Figure 1, and the results for the various bolus doses of NTG injection (0.01–0.3 mg/kg) are shown in Figure 2. The maximum depressive responses to the bolus NTG injections were comparable to the response to papaverine injection (5 μmol/kg), a NO-independent relaxant. In the NTG group, the depressor response to bolus NTG injection was markedly weakened with each dose of bolus NTG injection (P<0.01), demonstrating nitrate tolerance. In the groups with coadministration of carvedilol (Carv20 and Carv100 groups), the depressor effects of bolus NTG injections exhibited significantly (P<0.05) greater recovery than those in the NTG group, except for bolus NTG injections of 0.01–0.1 mg/kg in the Carv20 group. The depressor effects at lower doses of bolus NTG injection (0.03 and 0.1 mg/kg) were significantly (P<0.05) stronger in the Carv100 group than in the Carv20 group.

Effects of Continuously Coadministered Carvedilol on Superoxide Production in the Aorta and Heart

At identical laser and photomultiplier settings, the fluorescence of superoxides was increased in both the aortic wall and heart sections from the NTG group compared with the Vehicle group (Figure 3). However, the overexpression of superoxides induced by continuous NTG infusion was attenuated in the Carv20 and Carv100 groups to a similar extent. Representative sections from the Carv20 group alone are shown in Figure 4 for comparison with the Vehicle and NTG groups.

Effects of Continuously Coadministered Carvedilol on Cardiac CYP1A1/1A2 Expression

Compared with the Vehicle group, cardiac CYP1A1/1A2 levels were significantly decreased in the NTG group (P<0.01; Figure 4). However, in the Carv20 and Carv100 groups, the decreased expression of CYP1A1/1A2 induced by continu-
Carvedilol Attenuates Nitrate Tolerance

Ours NTG infusion exhibited significant recovery (P<0.05).

Effects of Continuously Coadministered Carvedilol on Changes in Aortic P-VASP
Whereas total VASP levels were comparable in all groups (Figure 5A), the P-VASP/VASP ratio was significantly less in the NTG group than in the Vehicle group (Figure 5B). The decrease in P-VASP was significantly attenuated in the Carv20 and Carv100 groups compared with the NTG group (P<0.05), and the degree of attenuation was significantly greater in the Carv100 group than in the Carv20 group (P<0.05).

Discussion
The results of this study reveal that continuous coadministration of carvedilol attenuates free radical generation, prevents CYP depletion, maintains NO/cGMP pathway activity, and ameliorates nitrate tolerance. These findings are consistent with those of randomized clinical studies elucidating the protective antioxidant effect of carvedilol against the development of nitrate tolerance6,16 and with another study demonstrating that coadministration of carvedilol could reduce the frequency of NTG usage in patients with angina pectoris.25

Nitrate tolerance has continued to be a significant problem,13,26 because it readily develops, particularly when the commonly used NTG skin patches are continuously applied for transdermal administration. Various clinical studies have attempted to attenuate the development of nitrate tolerance; for example, with use of other types of organic nitrates,26–28 coadministration of other agents6,15,16,29 or use of different dosing regimens.14,30,31 Organic nitrates with different numbers of −ONO2 residues might be bioactivated with different propensities to metabolism by the enzymes responsible for this.26,28 Isosorbide mononitrate with 1 −ONO2 was found to induce the development of moderate nitrate tolerance, despite suppression of vascular superoxide production.27 Coadministration of angiotensin-converting enzyme inhibitors has been reported to improve endothelial dysfunction, but not to improve nitrate tolerance.15,29 Intermittent administration of organic nitrates with nitrate-free intervals has been demonstrated to avoid tolerance, but with several disadvantages, including lack of protection against angina and rebound ischemia during or after the nitrate-free periods.14,31 In comparison, coadministration of carvedilol could be considered a favorable way of ameliorating nitrate tolerance without

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Figure 6. Schematic diagram of a possible mechanism by which coadministration of carvedilol attenuates nitrate tolerance, focusing on cytochrome P450 (CYP). Whereas nitroglycerin (NTG) when given acutely dilates vessels through the NO-cGMP pathway with sufficient CYP, long-term administration of NTG yields various related responses, shown as red dotted arrows (nitrate tolerance). Increased peroxynitrite, the product of NO/superoxide reaction, strongly inactivates CYP and inhibits the NTG-induced vasorelaxation cascade. However, these changes associated with nitrate tolerance are attenuated by coadministration of carvedilol, which has antioxidant effects (shown as blue arrows). Subsequent production of peroxynitrite, as well as superoxide, is suppressed by the coadministered carvedilol, the CYP level is correspondingly maintained, and NO-cGMP pathway activity is preserved, finally resulting in attenuation of nitrate tolerance. GTP, guanosine triphosphate; cGMP, cyclic guanosine monophosphate; sGC, soluble guanylate cyclase; cGK-I, cGMP-dependent protein kinase-I; NO, nitric oxide.
these disadvantages.⁷,¹⁶

Within the past decade, understanding of the mechanisms of nitrate tolerance has advanced considerably and 2 pathways of bioactivation of organic nitrates have been investigated: a high-affinity path with ALDH-2,²⁰,²³,²⁶ and a low-affinity path with the CYP system.²¹,²² Some considered that CYP was considered clearly involved in NTG bioactivation, because inhibition of ALDH-2 by knockout or inhibitors did not completely eliminate the vasodilating capacity of NTG.²³,²⁶ Gutterman also noted that metabolic activation of exogenous nitrate compounds required ALDH-2 and CYP.²⁵ Whatever the contribution of each enzyme to nitrate tolerance, most studies have highlighted the important role of enhanced superoxide production in the development of nitrate tolerance and have concluded that escape from nitrate tolerance appears to be mediated by direct antioxidant effects.⁷,¹⁸,²⁶,²⁷,³³

The theory of superoxide contribution to both nitrate tolerance and escape via antioxidant effects appears to be explained by the CYP system, although the mechanism underlying nitrate tolerance cannot be explained with reference to a single enzyme alone. We previously demonstrated that nitrate tolerance was the result of decreased CYP level and activity,²³,²⁴ and that deficiency of the antioxidant, vitamin E, induced nitrate tolerance because of a decreased level of CYP with impairment of NO/cGMP pathway activity.²⁵ A randomized clinical study has yielded findings consistent with this theory, demonstrating that supplemental vitamin E attenuates superoxide production and the development of nitrate tolerance.³³ Compared with vitamin E, carvedilol and its metabolites reportedly exhibit more potent antioxidant activity,³⁶ so may be a favorable agent for preventing nitrate tolerance.

Summing up our observations, a possible mechanism of the prevention of nitrate tolerance by coadministration of carvedilol based only on the CYP system is outlined in Figure 6. When NTG is acutely administered, the NO-cGMP cascade functions and finally mediates vasorelaxation via the generation of P-VASP, which has already been demonstrated as a sensitive monitor of NO/cGMP signaling.²⁴ However, when NTG is continuously administered, as observed in the present NTG group, vascular superoxide production become excessive (Figure 4), and the increased superoxide can be converted to peroxynitrite by reaction with NO.³⁶ The increased level of peroxynitrite, which is unstable with a half-life <1 s and reported to contribute to nitrate tolerance,³⁶ can inactivate CYP (Figure 3) and inhibit the NO-cGMP cascade (Figure 5B), although NO also inactivates CYP to a slight extent. When carvedilol is coadministered, it attenuates the degradation of CYP (Figure 3), suppresses superoxide production (Figure 4), and maintains the NO-cGMP cascade (Figure 5B), resulting in hemodynamic attenuation of nitrate tolerance (Figures 1, 2).

Study Limitations
First, vasodilating capacity differs according to location in the arterial circulatory system (ie, central or peripheral), and peripheral arteries are believed to principally determine systemic vascular resistance and blood pressure. However, in rats, the peripheral arteries are too small for sampling to evaluate levels of useful biomarkers. In our study the central portion of the aorta in the thorax and abdomen was the only adequate choice, and yielded a representative and appropriate sample. Second, attenuation of nitrate tolerance cannot be explained with reference to 1 enzyme alone, though the CYP system appeared to be acceptable and feasible for evaluation in our animal model, particularly in the case of high-dose, continuous NTG administration.²³ Third, carvedilol and NTG might compete for binding with the enzymes responsible for their degradation, because the biotransformation of carvedilol is also primarily mediated by CYP isoforms.³⁷ However, various CYP isoforms, such as CYP2D6, CYP2E1, CYP2C9, CYP3A4, and CYP1A2, have been reported to affect the disposition of carvedilol, and each of the isoforms was found to have limited effects on the degradation of carvedilol.³⁷ Furthermore, the CYPs responsible for the degradation of NTG and carvedilol may exist in different locations: the vasculature and liver, respectively. Interaction between NTG and carvedilol thus did not appear to have altered our findings. Fourth, this study did not examine the blood concentration of carvedilol required to attenuate nitrate tolerance, and could not determine the dose of carvedilol needed to attenuate nitrate tolerance in patients. Fifth, the present results could not clarify whether a β-adrenergic blocking effect alone or another antioxidant agent attenuated nitrate tolerance. Sixth, this study used normal rats only, so a further study using animal models such as those with hypertension will be necessary to discuss the clinical implications.

Conclusion
In conclusion, we have elucidated an aspect of the mechanism of prevention of nitrate tolerance by coadministration of carvedilol, focusing in particular on the CYP system. Our findings demonstrates that carvedilol prevents CYP depletion in the cardiovascular system and attenuated nitrate tolerance via antioxidant effects, consistent with the findings of previous randomized clinical studies.⁷,⁶ Organic nitrates are still important agents with strong efficacy in relieving the symptoms of angina and reducing cardiac preload and afterload, and a large proportion of patients taking organic nitrates are candidates for an additional β-blocker agent for management of deterioration of cardiac function or hypertension. Owing to the favorable effect of additional carvedilol to attenuate nitrate tolerance, it is the preferable choice of β-blocker agent in patients taking organic nitrates. In addition, the present findings may aid elucidation of the multifactorial mechanism of nitrate tolerance.

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Conflict of interest: none declared.

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References


