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Anti-NO action of carvedilol in cell-free system and in vascular endothelial cells

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1 Carvedilol, an adrenoceptor blocker with antioxidant activity, was studied for its ability to interact with NO in a cell-free condition and in an endothelial cell line (ECV304).

2 In a cell-free system, carvedilol attenuated NO-dependent reduction of carboxy-2-phenyl-4,4,5,5-tetramethyl-imidazoline-1-oxyl-3-oxide induced by a NO donor, 1-hydroxy-2-oxo-3-(aminopropyl)-3-isopropyl-1-triazene (NOC5), which was determined by electron paramagnetic resonance (EPR) spectrometry. The EPR study also showed that nitrosylhaemoglobin formation in rat red blood cells by the addition of NO-saturated solution was attenuated by prior incubation with 0.1–10 μM carvedilol.

3 NO-induced fluorescence in 4,5-diaminofluorescein-2 diacethyl (DAF-2DA)-loaded ECV304 cells was attenuated by carvedilol but not by labetalol. The IC_{50} of carvedilol for NOC5 or sodium nitroprusside-induced fluorescence of DAF-2DA in ECV304 cells was 1.0×10^{-7} M, which was similar to the reported IC_{50} of carvedilol for the antioxidant effect.

4 Cell toxicity induced by a NO donor determined by the number of viable cells after 24 h treatment with 2-2′(hydroxynitrosohydrazino)bis-ethanamine was significantly attenuated by pretreatment with 1 μM carvedilol.

5 Both free and cell-associated carvedilol quenched NO. Because NO mediates both physiological and pathophysiological processes, NO quenching by the drug may have diverse clinical implications depending upon specific functions of local NO in tissues where carvedilol is distributed.

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Abbreviations: cPTIO, carboxy-2-phenyl-4,4,5,5-tetramethyl-imidazoline-1-oxyl-3-oxide; DAF-2DA, 4,5-diaminofluorescein-2 diacethyl; DMEM, Dulbecco's modified eagle medium; D-PBS, Dulbecco's phosphate buffered saline; EPR, electron paramagnetic resonance; HBSS, Hank's balanced salt solution; NO, nitric oxide; NOC5, 1-hydroxy-2-oxo-3-(aminopropyl)-3-isopropyl-1-triazene; NOC18, 2-2′(hydroxynitrosohydrazino)bis-ethanamine; NOR1, (±)-(E)-4-methyl-2-[(E)-hydroxyimino]-5-nitro-6-methoxy-3-hexenamide; SNP, sodium nitroprusside

Introduction

Nitric oxide (NO) is a free radical which reacts with various molecules to cause multiple biological effects (Cohen, 1999; Grisham *et al*., 1999). In a biological system, NO may be produced enzymatically or nonenzymatically (Grisham *et al*., 1999; Weitzberg & Lundberg, 1998; Zweier *et al.,* 1995). While the molecule mediates many physiological functions, an excessive presence of NO is toxic to cells. High concentrations of NO in biological systems may be caused by NO-producing cells such as activated macrophages or by pharmacological manipulations such as administration of NO-donor.

The molecules that react with NO include oxygen-derived free radicals, thiols, and transitional metals, and some free radical scavengers (Gow & Stamler, 1998; Grisham *et al*., 1999; Moretensen *et al*., 1997; Yoshioka *et al*., 1996). One of the free radical scavengers currently used in the clinical setting is carvedilol which is a blocker of α1- and β-adrenoceptors (Yue *et al*., 1992). Becaues of its unique ability to interact with free radicals, carvedilol has been proposed to be a useful adrenergic antagonist in the treatment of hypertension and cardiac diseases in which oxidant stress prevails (Feuerstein *et al*., 1998). It has been postulated that carvedilol may increase local NO concentration by eliminating free radicals which interact with NO (Packer *et al*., 1996; Feuerstein *et al*., 1998). However, a direct interaction between carvedilol and NO has not been reported. In the present study, the interaction of NO and carvedilol was examined *in vitro* using electron paramagnetic resonance (EPR) spectrometry. The effect of carvedilol to alter intracellular NO concentrations was determined by fluorometric detection of NO in a human umbilical vein endothelial cell line. The functional significance of carvedilol in modifying cellular toxicity induced by NO was also evaluated.

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Methods

Drugs and chemicals

Carvedilol was provided by Dai-Ichi Pharmaceutical Co. Ltd. (Tokyo, Japan) and was dissolved 5% DMSO (Sigma, St. Louis, MO, U.S.A.) in 5 mN HCl (Wako, Osaka, Japan). Labetalol was obtained from Sigma and dissolved in the above solvent. Medium 199, Dulbecco's modified eagle medium (DMEM), Hank's balanced salt solution (HBSS), Dulbecco's phosphate buffered

saline (D-PBS), RPMI 1640, foetal bovine serum, amphotericin B, and penicillin-streptomycin were from Gibco BRL (Rockville, MD, U.S.A.). High grade carboxy-2-phenyl-4,4,5,5-tetramethyl-imidazoline-1-oxyl-3-oxide (c-PTIO), 1-hydroxy-2-oxo-3-(aminopropyl)-3-isopropyl-1-triazene (NOC5), 2-2′(hydroxy-nitroso-hydrazino)bis-ethanamine (NOC18), (±)-(E)-4-methyl-2-[(E)-hydroxyimino]-5-nitro-6-methoxy-3-hexenamide (NOR1) were from DOJINDO Laboratories (Kumamoto, Japan); 4,5-diaminofluorescein-2 diacethyl (DAF-2DA) from Dai-ichi Kagaku (Tokyo, Japan); alamar blue from Serotec (Kidlington, U.K.), and all other reagents from Sigma.

Determination of NO concentration by EPR spectrometry

The ability of carvedilol to quench NO *in vitro* was studied by EPR spectrometry. For this experiment, 100 μM c-PTIO in HBSS containing 10 μM carvedilol or the solvent was incubated with 10 μM NOC5 for 40 min. cPTIO has been shown to be reduced to cPTI specifically by NO to give specific EPR signals. NOC5 is a NO donor with a half life of approximately 25 min (Akaike *et al*., 1993; Iwamoto *et al*., 1996). At the end of the incubation, 200 μl of the solution was transferred to a quartz sample tube for subsequent analysis using an EPR spectrometer (JES-RE1X, JOEL, Akishima, Japan) at room temperature. The settings for the EPR measurement were: microwave frequency 9.42 GHz, microwave power 8.0 mW, time constant 0.1 msec, sweep time 60 s, centre field 335.8 mT, scan range 10 mT, modulation frequency 100 kHz, field modulation width 0.063 mT, and receiver gain $\times 500$ (Iwamoto *et al*., 1996). Concentrations of carvedilol used in the present study did not affect the EPR signal of cPTIO.

Another EPR study was performed to evaluate if carvedilol-loaded cells are protected from exogenous NO directly applied to cells *in vitro*. For this purpose, red blood cells (RBC) were used because haemoglobins contained in the RBC form a stable NO adduct, nitrosylhaemoglobins, which are detectable by EPR (Yoshioka *et al*., 1996). The nitrosylhaemoglobin (NO-Hb) exhibits a specific EPR spectrum of $g=2.04$ at 77°K (Henry *et al*., 1993). In this study, heparinized venous blood was obtained from Sprague-Dawley rats (Charles-River Japan, Tokyo, Japan), and was centrifuged at $800\times g$ at 4°C. The RBC were washed twice with degassed D-PBS, incubated with carvedilol (0.1 – 100 μM) or vehicle for 2 h on ice, and washed twice with D-PBS. The final wash fluid had no NO-quenching activity determined by the EPR using c-PTIO. Six μl of NO-saturated HBSS were then added to 600 μl of RBC suspension (haemoglobin concentration was 70 mg ml^{-1}). The NO-saturated HBSS was prepared by bubbling pure NO gas in HBSS placed in a hypoxic chamber for 60 min. The EPR spectrum of Hb was obtained at 77°K (in liquid nitrogen) using the following EPR settings: microwave frequency 9.02 GHz, microwave power 4.0 mW, time constant 0.3 msec, sweep time 240 s, centre field 330.0 mT, scan range 500 mT, modulation frequency 100 kHz, field modulation width 0.63 mT, and receiver gain $\times 500$. The EPR signal of nitrosylhaemoglobin was double integrated to calculate the concentration using $CuSO_4$ as standard (Yoshioka *et al*., 1996).

Ability of cell-associated carvedilol to quench NO

The ability of carvedilol to reduce intracellular NO was studied in a human umbilical vein endothelial cell line (ECV304) (Amanuma & Mitsui, 1991; Takahashi *et al*., 1990). ECV304 cells were propagated in M199 medium containing 10% fetal bovine serum, 100 μ ml^{-1} penicillin, 100 μg ml^{-1} streptomycin and 2.5 μg ml^{-1} amphotericin B; and were incubated in a CO_2 incubator (5% CO_2, 37°C). NO detection was performed by fluorometric examination of cells loaded with DAF-2DA, a membrane permeable fluorescent indicator for NO. DAF-2 has been shown to form green-fluorescent trazolofluorescein by reacting with NO (Kojima *et al*., 1998). For the fluorometric experiments, cells were subcultured in a 24-well plate (Iwaki, Tokyo, Japan) to subconfluency. The cells were incubated with serum-free medium containing various concentrations of carvedilol (1×10^{-8} to 3×10^{-5} M) for 2 h, followed by 10 μM DAF-2DA in DMEM for 1 h. After washing twice with 100 mM PBS, pH 7.4, the cells were incubated with PBS containing sodium nitroprusside (SNP), NOC5, or vehicle (0.9% NaCl for SNP; and 0.1 N NaOH for NOC5) for 30 min. In some experiments, labetalol, which is also an adrenergic antagonist but lacks antioxidant potency, was tested. Cells were then washed with PBS and examined under an inverted fluorescent microscope (IXE70, Olympus, Tokyo, Japan) at a $\times 200$ magnification.

A digital image of the fluorescent microscope image was obtained with a CCD camera (Hamamatsu Photonics, Hamamatsu, Japan) and stored in a Macintosh G3 personal computer (Apple Japan, Tokyo, Japan). The intensity of fluorescence was determined by densitometry using digital analytical software (MacAspect, Mitani Co., Tokyo, Japan). For this purpose, the image of each cell was traced and the intensity of green fluorescence produced by DAF-2 was measured. To examine the dose-response effect of carvedilol and labetalol, the ratio of fluorescence of drug-treated to untreated cells was calculated as relative intensity.

Effect of carvedilol against NO-induced cellular toxicity

The potency of carvedilol to attenuate NO-induced injury in ECV304 cells was evaluated by determining viable cell number following exposure to NO. For this purpose, ECV304 cells were plated in a 96-well plate (Iwaki), cultured to 60% confluency, and were incubated in a serum-deprived medium for 48 h. The cells were treated with 1 μM carvedilol for 2 h followed by addition of different concentrations of NOC18, which releases NO with a half life of approximately 21 h. Twenty-four hours after treatment with NOC18, the medium was replaced with RPMI1640 containing 10% alamar blue. After 4 h of incubation, oxidation of the dye was determined by measuring difference of absorbance between 595 and 575 nm using a microplate reader (Model 550, Biorad, Hercules, CA, U.S.A.). The oxidation of alamar blue has been used as an index of viable cell number (Ahmed *et al*., 1994; Shahan *et al*., 1994).

Statistics

Values are expressed as mean ± s.d. where applicable. Unpaired *t*-test was used to compare two groups. Statistical significance was defined as $P<0.05$.

Results

Potency of free and cell-associated carvedilol to quench NO

The EPR study demonstrated that carvedilol reduced NO-dependent reduction of cPTIO to cPTI. As shown in Figure 1, 10 μM NOC5, the NO donor, changed the EPR signals from a 5-line to 9-line pattern, which are those of cPTIO and cPTI,

respectively. In the presence of 10–100 μM carvedilol, the formation of cPTI was markedly attenuated. The higher concentration of carvedilol completely abolished NOC5-induced reduction of cPTIO (Figure 1d). Similar results were obtained in the experiments using NOC1 as NO donor (data not shown). These results indicate that carvedilol is capable of scavenging NO in a cell-free condition.

Then, we evaluated if carvedilol protects nitrosylation of intracellular molecules when cells are exposed to NO. As previously demonstrated (Yoshioka *et al*., 1996), addition of NO-containing solution to RBC resulted in the appearance of the EPR signal of nitrosylhemoglobin (Figure 2a,b). When RBC were incubated with carvedilol prior to the application of NO-containing solution, there was a marked attenuation in the formation of nitrosylhemoglobin (Figure 2c). Because the cells were washed extensively to eliminate free carvedilol before addition of NO, the results suggested that the cell-bound carvedilol quenched NO to prevent nitrosylhemoglobin formation. Although not shown, carvedilol itself did not affect the EPR signal of hemoglobins. When concentrations of nitrosylhemoglobin were determined from the EPR signals, carvedilol dose-dependently attenuated nitrosylhemoglobin formation (Figure 2d).

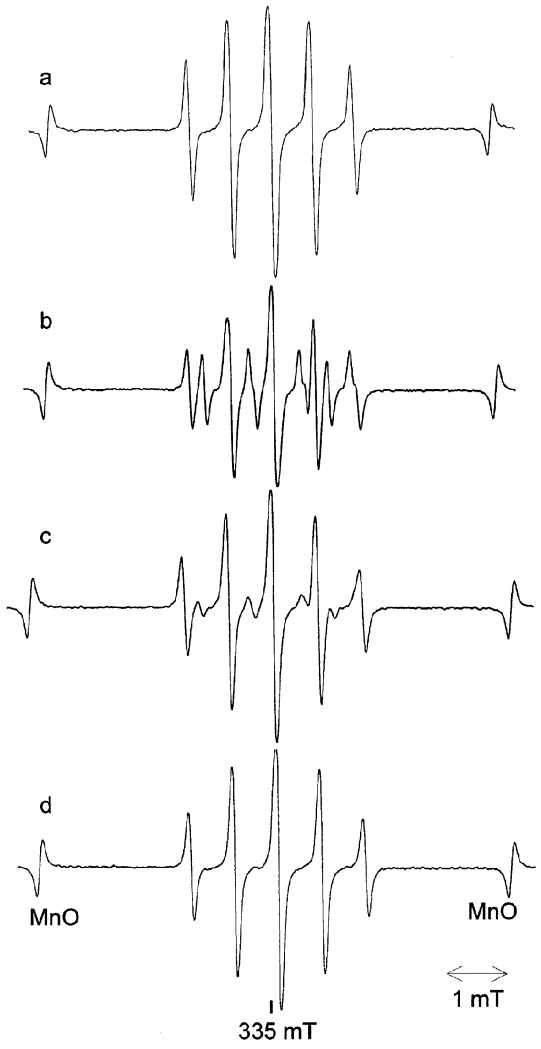

Figure 1 Effect of carvedilol on NO donor-induced reduction of cPTIO. The interaction of carvedilol and NO was examined in a cell free system using an electron paramagnetic resonance (EPR). (a) The EPR spectrum of cPTIO. cPTIO (100 μM) in HBSS gave a 5-line EPR signal as previously reported (Iwamoto *et al*., 1996). (b) The EPR spectrum of cPTIO reacted with a NO donor. When 10 μM NOC5 was added to the cPTIO solution, NO-dependent reduction of cPTIO resulted in the appearance of a 9-line EPR signal of reduced cPTIO (i.e., cPTI). (c) The EPR spectrum of cPTIO reacted with the NO donor in the presence of carvedilol. When 10 μM carvedilol was present in the reaction mixture, 10 μM NOC5 produced little change in cPTIO signal, indicating that the NO-dependent reduction of cPTIO was attenuated by carvedilol. (D) In 100 μM carvedilol, NOC5-induced reduction of cPTIO was completely abolished. The signals at the both ends of the spectrum (MnO) is an internal standard (Mn) for the equipment and analysis conditions.

Potency of carvedilol to quench NO in cultured ECV304 cells

The ability of cell-incorporated carvedilol to reduce intracellular NO was evaluated in ECV304 cells. In a pilot study, DAF-2DA-loaded ECV304 cells exhibited a dose-dependent increase in fluorescence in response to SNP (10^{-5}–10^{-3} M; data not shown) and NOC5 (10^{-5}–10^{-3} M), while hydrogen peroxide did not induce the fluorescence (Figure 3). The actual fluorescent microscopic images are shown in Figure 4. The untreated DAF-2DA-loaded ECV304 cells showed minimum fluorescence, while a strong fluorescence was observed in those treated with NOC5 or SNP. However, when the cells were preincubated with 1 μM carvedilol for 2 h prior to the addition of NO donor, the fluorescence induced by NOC5 or SNP was substantially attenuated (Figure 4). The relative intensity of fluorescence (ratio of the fluorescence of carvedilol-treated to non-treated cells) was measured from the digital images of the cells. Carvedilol dose-dependently attenuated intracellular NO produced by NOC5 (Figure 5a) or SNP (Figure 5b). The calculated IC_{50} of the NO quenching effect of carvedilol in these experimental conditions was 1.10×10^{-7} M and 1.13×10^{-7} M for NOC5 and SNP, respectively. The same effect of carvedilol was observed when DAF-2DA was loaded in cells followed by addition of carvedilol and a NO donor (data not shown). Thus, the effect of carvedilol cannot be

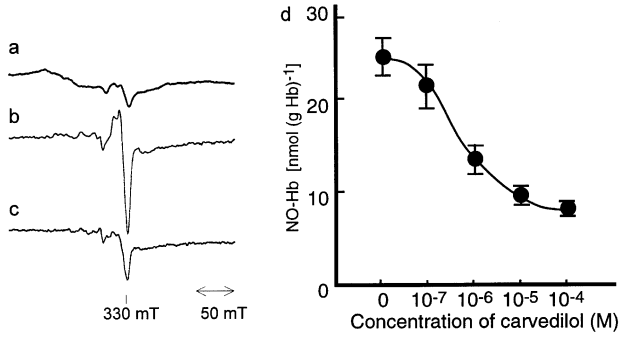

Figure 2 Attenuation of nitrosylhemoglobin formation by carvedilol in rat red blood cells (RBC) exposed to NO. The effect of cell-incorporated carvedilol on the formation of intracellular NO-haeme adduct was examined by EPR. (a–c) Rat RBC were suspended in PBS, pH 7.4. Six hundred μl of the RBC suspension was treated with the solvent (a mixture of 10% DMSO and 10% CH_3COOH, diluted 1000 times in PBS) (a), with the solvent followed by 6 μl of NO-saturated HBSS (b), and with 1 μM carvedilol followed by NO-saturated HBSS (c). EPR spectrometry was conducted in liquid nitrogen as previously described. The magnitude of the typical nitrosylhemoglobin signal at $g=2.04$ shown in (b) is markedly attenuated in (c). Although not shown, carvedilol (1–100 μM) did not affect the EPR signal of haemoglobin. (d) Rat RBC were treated with various concentrations of carvedilol followed by addition of NO-saturated HBSS as described above. The EPR signal of nitrosylhemoglobin was double integrated to calculate the concentration using $CuSO_4$ as a standard. Values are mean$\pm$s.d. of four determinations.

attributed to a decrease in cellular uptake of DAF-2DA. In contrast, labetalol showed no attenuation of NO levels in the cells treated with NOC5 (Figure 5a).

Effect of cell-incorporated carvedilol against NO-induced cell toxicity

Excessive NO has been shown to be toxic to living cells. The effect of carvedilol to attenuate NO-induced cellular injury was assessed by determining an index of viable cell number following exposure to NO. As shown in Figure 6, increasing doses of NOC18 caused a dose-dependent decrease in viable ECV304 cells determined at 24 h of treatment. Carvedilol significantly attenuated the effect of NOC18. These results suggested that NO quenching by carvedilol reduced the NO toxicity.

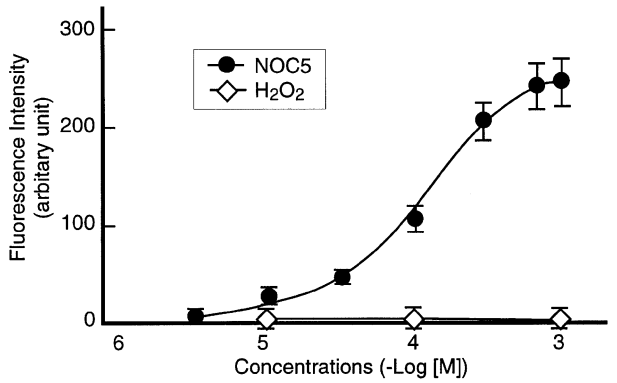

Figure 3 Fluorometry of DAF-2DA-loaded HUVEC treated with NOC5. ECV304 cells grown in a 48-well plate was incubated with 10 μM DAF-2DA in PBS, pH 7.4 for 60 min and washed twice with 100 mM PBS, pH 7.4. The cells were treated with given concentrations of NOC5 or hydrogen peroxide for 30 min and fluorometry was performed. The density of green fluorescence was measured by analysing the digital images using a computer as described in the Methods. Values are mean $\pm$ s.d. of 16–20 determinations.

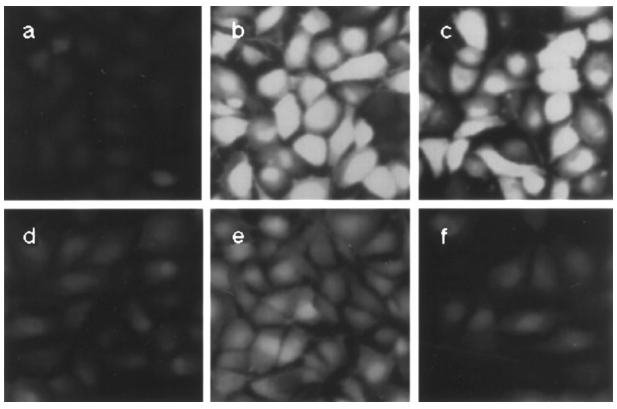

Figure 4 Fluorescence microscopy of the DAF-2DA-loaed ECV304 cells treated with NO donors and carvedilol. (a) ECV304 cells were loaded with DAF-2DA and fluorescent microscopy was performed. The unstimulated cells gave little fluorescence. (b–d) When DAF-2DA loaded cells were stimulated with 1×10^{-4} M NOC5 (b) or 1×10^{-4} M sodium nitroprusside (SNP) (c) for 30 min, the cells showed a marked increase in fluorescence, while 1×10^{-4} M hydrogen peroxide (d) showed no effect. (e,f) The ECV304 cells pretreated with 1 μM carvedilol showed marked attenuation in fluorescence induced by NOC5 (e) and SNP (f).

Discussion

Pharmacological properties of carvedilol as an adrenoceptor antagonist and an antioxidant have been described (Feuerstein *et al*., 1998; Kramer & Weglicki, 1996). The present study demonstrated a novel effect of carvedilol as a NO quenching agent. The results indicate that carvedilol directly interacts with NO in a cell-free system. Because of the lipophilic nature of the drug, the ability of cell-associated carvedilol to reduce NO was tested. Cell-associated carvedilol protected nitrosylation of intracellular molecule by exogenous NO and reduced intracellular concentration of NO produced by NO donors. The IC_{50} of carvedilol to quench NO produced by NOC5 and SNP was approximately 1×10^{-7} M in the cell culture study. The value is compatible with that reported for the antioxidant and cell protective effects of carvedilol ($0.1-2 \times 10^{-6}$ M) (Lysko *et al*., 1998). The rate of association of carvedilol and NO remained to be studied. Previous studies suggest an indirect effect of carvedilol to increase local NO concentration by eliminating free radicals that react with NO (Feuerstein *et al*., 1998), while our results were rather contrary to these suggestions. One reason for the discrepancy may be the difference in experimental conditions. The present study was designed to evaluate the direct interaction between NO and

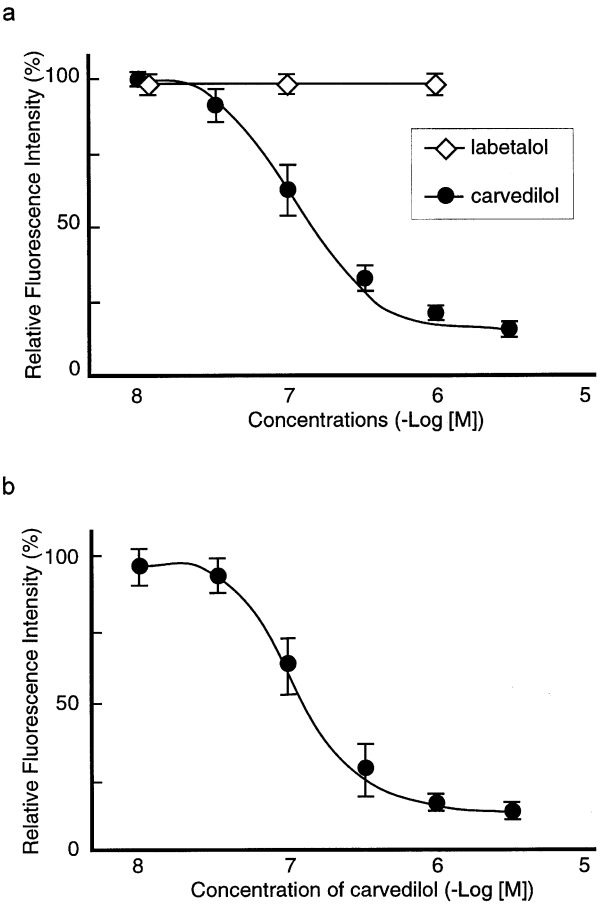

Figure 5 Fluorometric evaluation of NO production by ECV304 cells treated with NO donors, carvedilol and labetalol. ECV304 cells were incubated with different concentrations of carvedilol or with labetalol for 2 h, loaded with DAF-2DA and fluorescent images were obtained 30 min after treatment with 1×10^{-4} M NOC5 (a) or 1×10^{-4} M SNP (b). The intensity of green fluorescence was determined and the relative intensity to that of DAF-2DA-loaded cells treated with 1×10^{-4} M NOC5 (a) or of 1×10^{-4} M SNP (b) was calculated. Values are mean $\pm$ s.d. of 20 determinations from three separate experiments.

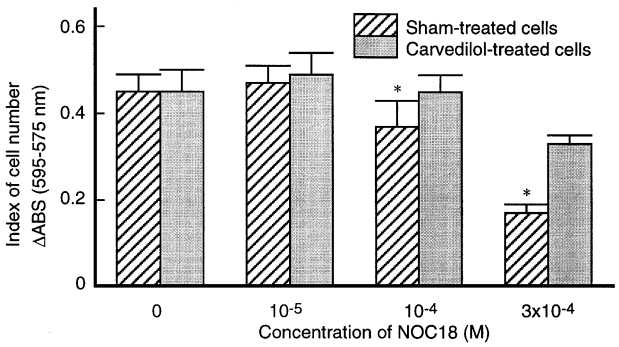

Figure 6 Effect of carvedilol on the number of viable ECV304 cells after exposure to NO. ECV304 cells plated on a 96-well plate were incubated with 1 μM carvedilol or vehicle for 2 h followed by treatment with given concentrations of NOC18. The numbers of viable cells were estimated 24 h later by the oxidation of alamar blue determined by the difference of absorbance between 575 and 595 nm. The number of cells incubated for an identical period without any treatment was used as a reference to calculate the relative number of treated cells to untreated control cells. $n=8$ ea. *Denotes $P<0.05$ between groups.

carvedilol, therefore, intervention by oxygen-derived free radicals is likely to be minimal. In the clinical setting, the effects of carvedilol on NO may be diverse depending on the local concentrations of free radicals and NO. Our results demonstrated that high concentrations of NO is effectively eliminated by the drug. Thus, carvedilol may function as an anti-NO agent at least in conditions where high biological concentrations of NO prevail.

The clinical implications of the present study also may be important. NO mediates both physiological and pathophysiological processes depending upon the source and concentrations of NO (Cohen, 1999). Adsorption of NO by carvedilol may not be beneficial for the antihypertensive effect because NO functions as a vasodilator. However, there may be situations in which high concentrations of oxidants and NO cause tissue injuries. The pharmacological characteristics of carvedilol may be preferable in such pathophysiological conditions. Nevertheless, the pharmacological effects of carvedilol need to be reevaluated with regard to its antioxidant effect in such conditions because NO is another free radical which may coexist with oxygen-derived free radicals.

The molecular mechanism for the NO quenching potency of carvedilol remains to be determined. The proposed antioxidant mechanisms of carvedilol include: (1) direct interaction with oxygen radicals; (2) prevention of the depletion of intracellular antioxidants; (3) attenuation of iron-mediated free radical formation (Yue *et al*., 1992; Franconi & Tadolini, 1998). Carvedilol has been shown to reduce the EPR signals formed by the oxygen radical generating systems. We have shown that several non-specific free radical scavengers adsorb NO in a cell-free NO generating system (Yoshioka *et al*., 1996; Iwamoto *et al*., 1996). The present study demonstrated that carvedilol reduced EPR signals of NO in a cell-free NO generating system. Thus, direct trapping of NO may constitute, at least in part, the anti-NO mechanism of carvedilol. Previous study have shown that other β-adrenoceptor antagonists have little antioxidant effect (Yue *et al*., 1992). The results using labetalol in the present study also suggest that the anti-NO effect is unique to carvedilol. An interaction with other antioxidants as well as with ferrous or ferric irons may also be a part of the anti-NO mechanisms, although these were not tested in the present study.

Our study described a novel pharmacological potency of carvedilol. As stated, this anti-NO action may have clinical implications which require further investigations. The metabolite of carvedilol (SB211475), which differs from carvedilol by being hydroxylated at the 3-position of the carbazole ring, has been shown to have stronger antioxidant potency than carvedilol (Kramer & Weglicki, 1996; Lysko *et al*., 1998). Thus, both carvedilol and SB211475 are interesting reagents to be tested *in vivo* for their ability to modify NO-mediated pathophysiological conditions.

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