Carvedilol Inhibits Mitochondrial Oxygen Consumption and Superoxide Production During Calcium Overload in Isolated Heart Mitochondria

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Background  The COMET study suggested the better effect of carvedilol to metoprolol in treating heart failure. However, its underlying mechanisms of action remain unclear. As a result, evaluation of the distinct effects of both drugs on the mitochondrial function and reactive oxygen species (ROS) production during Ca\textsuperscript{2+} overload was investigated.

Methods and Results  The mitochondrial oxygen consumption (m\textsubscript{VO}\textsubscript{2}) and the mitochondrial ROS production in isolated rat heart mitochondria was measured. Ca\textsuperscript{2+} overload from 10 to 100 \textmu mol/L augmented m\textsubscript{VO}\textsubscript{2} was from 327±139 to 671±138 nmol/mg (p<0.05), and this was then completely suppressed by carvedilol (1 \textmu mol/L), but not by metoprolol (100 \textmu mol/L). Ca\textsuperscript{2+} overload augmented the ROS production upon complex I injury (9.7±1.2 to 11.4±1.4 nmol/mg, p<0.05). Carvedilol dose-dependently suppressed this ROS production, whereas metoprolol did not.

Conclusions  Carvedilol, but not metoprolol, was thus found to inhibit the calcium-dependent augmentation of m\textsubscript{VO}\textsubscript{2} and ROS production upon complex I injury. This new effect of carvedilol might partly explain the beneficial effect of carvedilol for the treatment of heart failure.  (Circ J 2006; 70: 321–326)

Key Words: Calcium overload; Carvedilol; Mitochondria; Oxygen consumption; Superoxide

In the failing myocardium, calcium overload has been shown to play an important role in causing a dysfunction in the myocardium, and the reactive oxygen species (ROS) are produced from impaired mitochondria, which thus further impairs the cellular function. Betadrenergic receptor blockers have been shown to be effective for interrupting this malignant cycle of heart failure, thus attenuating both the calcium overload and the mitochondrial dysfunction. However, a recent clinical study, COMET, suggested that carvedilol had a better effect on the survival rate in patients with chronic heart failure than metoprolol. This study proposed that carvedilol, but not metoprolol, was able to inhibit the calcium-dependent augmentation of m\textsubscript{VO}\textsubscript{2} and ROS production upon complex I injury. This new effect of carvedilol might partly explain the beneficial effect of carvedilol for the treatment of heart failure.

Methods

Animals  Male Sprague–Dawley rats (7–9 weeks old, 300–350 g) were anesthetized with the intra-peritoneal injection of sodium pentobarbital (60 mg/kg) and then the heart was excised and rinsed with an ice-cold buffer containing 300 mmol/L mannitol, 10 mmol/L HEPES, 0.2 mmol/L EDTA, and 0.1% bovine serum albumin (BSA). The pH was adjusted to 7.4 with KOH. The hearts were trimmed and homogenized with a motor-driven Teflon Potter homogenizer for 1 min in an ice-cold buffer.

Isolation of Heart Mitochondria  Mitochondria were then isolated by the centrifuge method, as previously described with slight modifications. The heart homogenate was centrifuged at 700 G for 10 min at 4°C using the KUBOTA 3780 centrifuge. The supernatant was decanted and centrifuged at 5,000 G for 8 min, followed by 10,000 G for 5 min. The pellet was resuspended using a paint brush and then it was centrifuged at 2,500 G for 10 min, followed by 9,000 G for 10 min. EDTA was omitted from the final washing buffer. The mitochondrial protein concentration was determined by the Bradford method using BSA as a standard. The mitochondrial suspension (4 mg/ml) was kept on ice until the measurements were performed, which were carried out after a 20-min recovery. All experiments conformed to the Guide for the Care and Use of Laboratory Animals (NIH Publication No.85-23) and the protocol was approved by the Animal Research Committee of Yamaguchi University, School of Medicine.
Carvedilol was a gift from Daiichi Pharmaceutical (Tokyo, Japan) and it was dissolved in dimethyl sulfoxide (DMSO). The final concentration of DMSO was less than 0.1% when used. Metoprolol was purchased from Sigma and it was dissolved in normal saline.

**Measurement of Mitochondrial Oxygen Consumption (mV/\text{O}_2)\)**

\(\text{mV/O}_2\) was measured using a Clark-type electrode (Instech Laboratories, Plymouth Meeting, PA, USA) in a 600-\(\mu\text{L}\) sealed glass chamber with water-jacketed and maintained at 37°C, with constant magnetic stirring. The oxygen tension in the chamber was monitored by the electrode connected to PowerLab Systems (ADInstruments, Colorado Springs, CO, USA). Mitochondria at a concentration of 0.2 mg/ml were incubated in the chamber with a medium consisting of 50 mmol/L HEPES. The reactions were initiated by adding NADH to a final concentration of 4 mmol/L. Carvedilol or metoprolol was added to the reaction medium before adding NADH. At 2 min after NADH was added, CaCl\(_2\) was added to increase the calcium concentrations from 10 to 100 \(\mu\text{mol/L}\). The oxygen uptake was calibrated according to the oxygen consumed by mitochondria after addition of titrated solutions of NADH\(^{11,12}\)

**Measurement of ROS Production From Mitochondria**

Two segments of the respiratory chain are primarily responsible for producing ROS in the mitochondria: namely NADH-ubiquinone reductase in complex I and biquinol-cytochrome \(c\) reductase in complex III (Fig 1).\(^{13,14}\) ROS production was measured by lipid peroxidation, which was indicated by the amount of thiobarbituric acid reactive substance (TBARS).\(^{15}\) The mitochondrial suspension (0.2 mg/ml) was incubated at 37°C in 600 \(\mu\text{L}\) HEPES buffer. Then either carvedilol or metoprolol was added 3 min before measuring the lipid peroxidation, which was initiated by the addition of NADH (4 mmol/L), CaCl\(_2\) (10, 100 \(\mu\text{mol/L}\)), rotenone (100 \(\mu\text{mol/L}\)), and ADP/FeSO\(_4\) (2 mmol/L/0.2 mmol/L) were added just before initiating lipid peroxidation. After 15 min, the samples were taken and mixed with 1.9 ml of TBARS reagent (0.75% thiobarbituric acid, 5 mol/L HCl, 10 mmol/L butylated hydroxytoluene). The mixture was heated at 80°C for 15 min, and then was cooled on ice for 10 min before centrifugation (400G, 10 min). Lipid peroxidation was estimated by the appearance of TBARS with spectrophotometry quantified at 535 nm. The amount of TBARS was calculated using a molar extinction coefficient of 1.56\(\times\)10\(^5\) (mol/L)\(^{-1}\)cm\(^{-1}\) and then it was expressed as nano moles of malondialdehyde.

**Fig 1.** A schematic drawing of the components of the respiratory chain in mitochondria and the source of superoxide production. Rotenone inhibits complex I and the possible influence of Ca\(^{2+}\) overload is thus indicated. FAD, flavin adenine nucleotide; FMN, flavin mononucleotide; Fe-S, iron-sulfur protein; Q, ubiquinone; Cyt, cytochrome.

**Fig 2.** Mitochondrial \(\text{O}_2\) consumption (mV/\text{O}_2) was significantly augmented by increasing Ca\(^{2+}\) from 10 to 100 \(\mu\text{mol/L}\). Carvedilol (1 \(\mu\text{mol/L}\)) significantly suppressed the augmentation, whereas metoprolol did not. The data are expressed as the means ± SE, n=8 for each group.
Carvedilol and Mitochondrial ROS Production

Statistics

The data are expressed as the means±SE. A statistical analysis was performed using one-way ANOVA followed by the multiple comparison Scheffe test. Differences with a value of p<0.05 were considered to be significant.

Results

Mitochondrial O2 Consumption

Fig 2 showed mVO2 15 min after the addition of NADH. The mVO2 level significantly increased after adding Ca2+ to increase it from 10 to 100 μmol/L (p<0.05). A low dose of carvedilol (1 μmol/L) significantly reduced the augmented (MDA) per mg of protein.15

Fig 3. The dose-response effect of carvedilol and metoprolol on mitochondrial O2 consumption during Ca2+ overload (3A: 10 μmol/L, 3B: 100 μmol/L). The data are expressed as the means±SE, n=8 for each group. mVO2, mitochondrial O2 consumption.

Fig 4. The effect of rotenone (100 μmol/L) on the malondialdehyde (MDA) production during Ca2+ overload ranging from 10 to 100 μmol/L. The data are expressed as the means±SE, n=8 for each group.
mVO₂ under Ca²⁺ overload (100 μmol/L). However, metoprolol (1 μmol/L) did not suppress the mVO₂ during Ca²⁺ overload. Fig 3A shows the dose-response effect of carvedilol and metoprolol on mVO₂: under calcium overload (10 μmol/L). Carvedilol dose-dependently reduced mVO₂, but even 100 μmol/L metoprolol did not affect the mVO₂ level. Fig 3B shows the effect of carvedilol and metoprolol on mVO₂ under high Ca²⁺ overload (100 μmol/L). Even under a high Ca²⁺ overload, carvedilol dose-dependently reduced mVO₂, however even higher doses of metoprolol (100 μmol/L) did not affect the mVO₂ level.

Mitochondrial ROS Production During Ca²⁺ Overload

Fig 4 shows the amount of MDA produced 15 min after the addition of NADH to the mitochondria. Without rotenone (100 μmol/L), the Ca²⁺ overload ranging from 10 to 100 μmol/L did not affect the MDA production. However, the MDA production increased significantly with rotenone, and a Ca²⁺ overload ranging from 10 to 100 μmol/L then further increased the MDA production from the mitochondria. Fig 5 shows the effect of carvedilol and metoprolol on the MDA production at doses ranging from 10 μmol/L (Fig 5A) to 100 μmol/L (Fig 5B) of Ca²⁺ overload outside the mitochondria. Carvedilol dose-dependently decreased ROS production under the Ca²⁺ overload at both 10 and 100 μmol/L. However, even higher doses of metoprolol (100 μmol/L) did not affect the ROS production under Ca²⁺ overload.

Discussion

The present study showed for the first time that carvedilol, but not metoprolol, inhibited the augmentation of mVO₂ caused by Ca²⁺ overload. Metabolic energy homeostasis is known to be maintained during changes in the cardiac workload, whereas Ca²⁺ plays an important role in mediating the signals for mitochondria to produce the required amount of adenosine triphosphate (ATP) needed to match the cardiac energy metabolism. The proper coupling between the ATP requirement of the cardiomyocytes and the ATP production by the mitochondria is supposed to be regulated by the Ca²⁺ entry from the cytosol to the mitochondria, but the precise mechanism which regulates the ATP production in the cardiac mitochondria according to the increased cellular demand remains unclear. In the present study, the Ca²⁺ outside of the mitochondria varied from 10 to 100 μmol/L in order to examine the direct effect of Ca²⁺ overload on the mVO₂. We showed that the mVO₂ was augmented by increasing the Ca²⁺ concentration outside the mitochondria. The increase in the mVO₂ caused by
the Ca\textsuperscript{2+} overload can be explained by the following 2 mechanisms: one is the augmentation of the electron transport system by Ca\textsuperscript{2+} entry into the mitochondria, which thus leads to the enhanced production of ATP. This is supported by evidence that Ca\textsuperscript{2+} activates the Fo/F1 ATPase activity, which synthesizes ATP in the cardiac mitochondria\textsuperscript{18,19}. The other mechanism is related to the uncoupling of the electron transport system, which did not increase ATP production\textsuperscript{20}. This uncoupling is characterized by the proton leak through the mitochondrial inner membrane, which results in the disruption of the H\textsuperscript{+} gradient formed across the inner mitochondrial membrane by the electron transport system. When the uncoupling phenomenon occurs because of the calcium overload\textsuperscript{21} despite of any increase in the oxygen consumption, the ATP synthesis did not increase accordingly\textsuperscript{22}.

**Effect of Carvedilol on Ca\textsuperscript{2+} Dependent m\textsuperscript{VO}_{2}**

Our result suggests that carvedilol suppresses m\textsuperscript{VO}_{2} directly without any signaling from the \(\beta\)-adrenergic receptor system. The downstream cascade of \(\beta\)-adrenergic receptor stimulation includes cAMP, protein kinase A, and several phosphorylated proteins including the L-type calcium channels, phospholamban, and ryanodine receptors\textsuperscript{23}. However, the signaling to the mitochondria from the \(\beta\)-adrenergic receptor pathway has not yet been well elucidated. The cytosolic Ca\textsuperscript{2+} most likely plays an important role in mediating the \(\beta\)-adrenergic receptor signals to the mitochondria in order to regulate the ATP production\textsuperscript{24}. As a result, carvedilol might act to inhibit the Ca\textsuperscript{2+} entry into the mitochondria. Carvedilol can also inhibit the augmented m\textsuperscript{VO}_{2} caused by Ca\textsuperscript{2+} overload through an inhibitory effect on the respiratory uncoupling. In addition, the protonophoretic characteristics of carvedilol might be involved in this mechanism\textsuperscript{25}. Because the direct effect of carvedilol on the mitochondria was examined in the present study, isolated mitochondrial experiments were thus carried out. Although the effect of antioxidant and anti-apoptotic effect on the in vitro isolated cardiomyocyte has been shown\textsuperscript{26,27} the direct effect of carvedilol on mitochondria during calcium overload has never been evaluated. The impact of the present study on the treatment of heart failure thus needs to be further clarified. By either mechanism, carvedilol was found to have a direct effect on the mitochondria thus resulting in the inhibition of the oxygen consumption during Ca\textsuperscript{2+} overload independent of the \(\beta\)-adrenergic receptor system\textsuperscript{28}.

**Mechanism of Augmented ROS Production by Ca\textsuperscript{2+} and Rotenone**

The present study found that the normal mitochondria isolated from the rat cardiomyocytes did not increase the ROS production as measured by the amount of MDA under Ca\textsuperscript{2+} overload. However, the mitochondria mimicking a state of heart failure by impairing complex I with rotenone (100 \(\mu\)mol/L) became sensitive to Ca\textsuperscript{2+} overload in order to increase the ROS production. This occurrence has also been observed for the forebrain mitochondria, but not for the isolated mitochondria from either liver or skeletal muscle\textsuperscript{29}. Rotenone was used as an inhibitor of complex I in a mitochondrial electron transport system, which is known to be impaired in a state of heart failure (Fig 1). In a state of heart failure, complex I has been shown to be a major source of ROS in the mitochondria\textsuperscript{8} and this rotenone specific domain has also been shown to be sensitive to Ca\textsuperscript{2+} in the production of ROS\textsuperscript{29}. As a result, carvedilol might act on this rotenone-sensitive domain of complex I in order to inhibit the ROS production caused by Ca\textsuperscript{2+} overload. In addition, these mechanisms can also be specific for the heart and forbrain, but not for the liver or skeletal muscle\textsuperscript{29}.

**Distinct Effects Between Carvedilol and Metoprolol**

We herein showed that the effect of carvedilol on the mitochondrial function, such as oxygen consumption and ROS production, is distinct from that of metoprolol. Both carvedilol and metoprolol are known to be potent \(\beta\)-adrenergic receptor blockers, which can thus suppress Ca\textsuperscript{2+} overload by stabilizing the calcium release channels (ryanodine receptors) on the sarcoplasmic reticulum\textsuperscript{3} and clinical trials of both drugs have shown these drugs to be effective in treating chronic heart failure\textsuperscript{29}. However, a recent study comparing the effect of carvedilol and metoprolol, called the COMET study\textsuperscript{7} indicated carvedilol to have a better efficacy in comparison to metoprolol regarding the event-free survival in heart failure patients. The present study further provides definite evidence that carvedilol differs significantly from metoprolol in inhibiting the Ca\textsuperscript{2+} overload-induced mitochondrial oxygen consumption and the ROS production during an impairment of complex I. Previous studies also support our results using different models\textsuperscript{26,31}. This distinct effect between the drugs might partly explain the results of the COMET study: ie, a \(\beta\)-adrenergic receptor independent effect\textsuperscript{27}. Importantly, carvedilol suppressed the production of ROS, especially during an impairment of complex I, which mimics a state of heart failure. The ROS production has been shown to be enhanced in a state of heart failure because of the \(\beta\)-adrenoceptor dependent pathway\textsuperscript{32}. As a result, it is conceivable that the \(\beta\)-adrenoceptor blockades reduced the ROS production through the \(\beta\)-adrenoceptor pathway\textsuperscript{33}. However, ROS could be produced by many different pathways such as the angiotensin II mediated pathways through the activation of NADPH oxidase and the TNF mediated pathways in patients with heart failure\textsuperscript{34,35}. The antioxidant effect of carvedilol might inhibit all ROS-induced toxic effect on both intracellular and mitochondrial calcium handling, because this effect is independent of the \(\beta\)-adrenoceptor pathway. Therefore, carvedilol might be more effective during the state of heart failure than under normal conditions\textsuperscript{36}. Finally, the rationale regarding the dose used in the present study needs to be discussed. The ID\textsubscript{50} for \(\beta\)-adrenoceptors might be lower in carvedilol than in metoprolol, but the ID\textsubscript{50} for \(\beta\)-adrenoceptor blocking effect is nanomolar order for both drugs. As a result, the micromolar order of the concentration used in the present study is sufficient to block \(\beta\)-adrenceptors by both drugs. Moreover, the antioxidant effect of the drugs might not necessarily coincide with the \(\beta\)-adrenoceptor blocking effect. Since the 100 \(\mu\)mol/L metoprolol did not show any effect in the present study, our conclusions will therefore not change regardless of the dose used.

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