Effects of Carvedilol and Its Analog BM-910228 on Mitochondrial Function and Oxidative Stress

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ABSTRACT

The antioxidant effects of carvedilol and its analog BM-910228 (also known as SB 211475) were studied in rat liver mitochondria as well as their action on mitochondrial bioenergetics. Carvedilol and BM-910228 inhibited ADP/Fe²⁺-initiated lipid peroxidation (measured in mitochondrial membranes as thio-barbituric acid reactive substances and oxygen consumption) with IC₅₀ values of 10.9 and 0.33 μM, respectively. Under the same conditions, the IC₅₀ value for Trolox C was 18.8 μM. At the same concentration range showing antioxidant activity both compounds prevent the collapse of transmembranar electric potential induced by ADP/Fe²⁺ on respiring mitochondria. Furthermore, both carvedilol and BM-910228 do not display toxic effects on mitochondria up to the concentration showing maximal antioxidant effects (~40 μM for carvedilol and ~1 μM for BM-910228). At higher concentrations of carvedilol (~40 μM), however, the phosphorylation efficiency of mitochondria is depressed as deduced from a decrease in respiratory control and in the ADP/oxygen ratio. The Brand approach was used to assess the effects of carvedilol on oxidative phosphorylation. We found that carvedilol stimulated membrane proton leak and inhibited substrate oxidation, but had no measurable effect on phosphorylation reactions. Because carvedilol exerts its antioxidant properties for nontoxic concentrations, its therapeutic interest is reinforced because it may potentially prevent mitochondrial dysfunctions associated to cell death in several pathophysiological states where excessive production of reactive oxygen species by mitochondria is well documented (e.g., ischemia/reperfusion). Additionally, its hydroxylated analog BM-910228 with notable superior antioxidant activity may significantly contribute to the known therapeutic effects of carvedilol.

Carvedilol is a lipophilic nonselective β-adrenoceptor blocker with vasodilator effects exerted primarily through selective α₁ receptor blockade (Dunn et al., 1997) and with a strong antioxidant effect (Yue et al., 1992). This drug was initially approved for treatment of mild to moderate hypertension. More recently, carvedilol has been shown to slow the progression of disease, to reduce the risk of death, and to improve the quality of life in patients with congestive heart failure and to improve the ischemic symptoms associated with angina (Dunn et al., 1997).

Carvedilol has been shown experimentally to decrease infarct size in several experimental models studied (Feuerstein et al., 1996) and to exert protective effects on several models of cardiovascular ischemia and reperfusion (Ma et al., 1996). The mechanisms underlying the multiple cardioprotective effects of carvedilol are not fully understood; however, the reported beneficial effects of carvedilol seem to be, partially, due to its potent antioxidant activity (Yue et al., 1992). Carvedilol was shown to inhibit lipid peroxidation in swine ventricular membranes (Yue and Feuerstein, 1992), rat brain homogenates (Yue et al., 1992), human low-density lipoproteins (Yue et al., 1995), bovine and human endothelial cells, as well as in modellar membranes (Tadolini and Franconi, 1998). The antioxidant protection of carvedilol was demonstrated to act through a chain-breaking mechanism in experimental models of postischemic rat hearts (Kramer and Weglicki, 1996). It is interesting to note that the capacity of carvedilol to inhibit lipid peroxidation is much greater than that of other tested β-blockers, such as propranolol, which may also explain the superior protective effects of carvedilol in ischemia/reperfusion models (Yue and Feuerstein, 1992). In fact, reactive oxygen species (ROS) have been implicated in myocardial damage during ischemic insults (Coetzee et al., 1990), and especially in the beginning of reperfusion (Downey, 1990).

Carvedilol is almost completely metabolized in vivo, remaining intact only 2% of a dose when excreted in the urine.

ABBREVIATIONS: ROS, reactive oxygen species; DMSO, dimethyl sulfoxide; RCR, respiratory control ratio; ΔVy, transmembrane electric potential; Δp, proton motive force; TPP⁺, tetraphenylphosphonium ion; TBARS, thiobarbituric acid reactive substances; MDA, malonic dialdehyde; FCCP, p-trifluoromethoxyphenylhydrazone; ATPase, ATP synthase.

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(Neugebauer et al., 1987). Among the various metabolites of carvedilol, BM-910228 [1-3-hydroxy carbazolyl-(4)-oxy-3-(2-methoxyphenoxo-ethyl)amino-propanol-(2)] is one of the most important, formed by introduction of a hydroxyl group at position 3 of carvedilol carbazole moiety. BM-910228 concentration in plasma is 10-fold less than the observed concentration for carvedilol. This metabolite has revealed a potent antioxidant activity at concentrations about 30 times inferior to carvedilol (Yue et al., 1992) being very effective in various lipid peroxidation models.

In some pathological conditions, and especially after ischemia and reperfusion, mitochondria are recognized to dramatically increase the production of ROS concomitant with the reduction of its antioxidant defenses (Ferrari, 1996). Cytotoxic effects of ROS are mediated by the peroxidation of lipid components of cellular and mitochondrial membranes. In particular, the presence of polyunsaturated fatty acid-rich membranes enhances mitochondrial susceptibility to lipid peroxidation, leading to membrane dysfunction and alterations on structural and functional integrity of mitochondria (Tien et al., 1981). Irreversible mitochondrial injury correlates with the inability to restore cellular functions and, ultimately, with cell death. Therefore, antioxidant action of carvedilol and its metabolite BM-910228 may contribute to the preservation of mitochondrial functions in several pathophysiological conditions.

The purpose of the present study was to investigate the in vitro antioxidant capacity of carvedilol and its metabolite BM-910228 on isolated liver mitochondria subjected to severe oxidative damage. We have further investigated the direct effects of these compounds on mitochondrial function to clarify whether the compounds have any toxic effects on mitochondria at the concentration range showing antioxidant capacity.

**Experimental Procedures**

**Animals.** Male Wistar rats (250–350 g), housed at 22 ± 2°C under artificial light for 12-h light/dark cycle and with access to water and food ad libitum, were used throughout the experiments. The experiments reported here were carried out in accordance with the National Requirements for Vertebrate Animal Research and in accordance with the European Convention for the Protection of Animals used for Experimental and other Scientific Purposes.

**Materials.** All chemicals used were of the highest grade for research. Carvedilol and BM-910228 were obtained from Boehringer Mannheim (Mannheim, Germany) and dissolved in absolute dimethyl sulfoxide (DMSO). Pure solutions of DMSO were added to controls with the highest volume used of both compounds’ DMSO solutions (0.1% of the experiments final volume) and had no effects on the measured activities.

**Isolation of Rat Liver Mitochondria.** Rat liver mitochondria were isolated by conventional differential centrifugation (Johnson and Lardy, 1967) from the livers of overnight-fasted adult male Wistar rats weighing 250 to 350 g, with slight modifications. Rats were decapitated, and their livers were harvested and rinsed with an ice-cold buffer containing 250 mM sucrose, 10 mM HEPES, 1 mM EGTA, and 0.1% defatted BSA. The pH was adjusted to 7.4 with KOH. Livers were trimmed and homogenized 1 min with a motor-driven Teflon Potter homogenizer in the presence of the ice-cold buffer (7 g/50 ml). Liver homogenate was centrifuged at 800 g for 10 min (Sorvall RC-5C, Plus, SS 34 rotor, 4°C) and its supernatant at 10,000 g for 10 min. The mitochondrial pellet was resuspended using a paintbrush and centrifuged twice at 10,000 g for 10 min before obtaining a final mitochondrial suspension. EGTA and defatted BSA were omitted from the final washing medium, which was adjusted to pH 7.2. Mitochondrial protein was determined by the biuret method (Gornall et al., 1949). Mitochondrial suspension (≈30–40 mg of protein/ml) was kept on ice before experiments, which were carried out after a 20-min recovery and within 5 h.

For lipid peroxidation assays, the mitochondrial pellet was pelleted in a buffer consisting of 175 mM KCl, 10 mM Tris, pH 7.4, to remove sucrose, which may interfere with the thiobarbituric acid reaction.

**Mitochondrial Oxygen Consumption Measurements.** Oxygen consumption was measured using a Clark-type oxygen electrode (Yellow Springs Instrument, Yellow Springs, OH) connected to a recorder (BD 112; Kipp & Zonen, Delft, The Netherlands) in a 1-ml thermostated, water-jacketed, sealed glass chamber with constant magnetic stirring, at 25°C. Reactions were conducted in a medium containing 130 mM sucrose, 50 mM KCl, 5 mM MgCl₂, 5 mM KH₂PO₄, 5 mM HEPES (pH 7.2), supplemented with 5 μM rotenone. The reactions were initiated by adding potassium succinate to a final concentration of 5 mM. State 4 respiration was monitored for at least 3 min before adding ADP (0.1 mM) to initiate state 3 respiration. Carvedilol or BM-910228 was added in DMSO solutions (up to 0.1% of the experiments final volume) to the reaction medium with mitochondria and allowed to incubate for 3 min. This incubation period was carried out to ensure the complete internalization of the compounds on the membrane due to their lipophilic characteristics. After the 3 min, succinate was added to induce state 4 condition. Care was taken to ensure a final assay volume of 1 ml after additions. Respiratory control ratios (RCR = state 3/state 4), respiratory states, and ADP/O ratios were determined according to Chance and Williams (1956). The scale of oxygen uptake was calibrated according to the oxygen consumed by submitochondrial particles after addition of titrated solutions of NADH.

**Measurements of Mitochondrial Transmembrane Electrical Potential (ΔΨ).** ΔΨ was monitored indirectly based on the activity of the lipophilic cation tetrathenylphosphonium (TPP⁺) using a TPP⁺-selective electrode prepared in our laboratory, in combination with a Ag/AgCl-saturated reference electrode (model MI 402; Microelectrodes, Inc., Bedford, NH) (Kamo et al., 1979). Both the TPP⁺ electrode and the reference electrode were inserted into the chamber used for determination of mitochondrial oxygen consumption and were connected to a pH meter (model 330S; Jenway, Essex, UK). The signals were simultaneously fed to a dual-trace potentiometric recorder (model BD 121; Kipp & Zonen). Mitochondria were incubated in the reaction medium (130 mM sucrose, 50 mM KCl, 5 mM MgCl₂, 5 mM KH₂PO₄, 5 mM HEPES, pH 7.2, supplemented with 3 μM rotenone) containing 3 μM TPP⁺. The reaction was stirred continuously and the temperature maintained at 25°C. The ΔΨ was estimated as indicated by Kamo et al. (1979) from the following equation (at 25°C): ΔΨ = 59 × log (v/V) − 59 × log (10²¹⁰⁻¹), where v, V, and ΔE stand for mitochondrial volume, volume of incubation medium, and deflection of the electrode potential from the baseline, respectively. A matrix volume of 1.1 μl/mg of protein was assumed. No correction was made for the “passive” binding of TPP⁺ to the mitochondrial membranes because the purpose of the experiments was to show relative changes in potentials rather than absolute values. As a consequence, we can anticipate some overestimation for the ΔΨ values. Neither carvedilol nor BM-910228 affect TPP⁺ binding to mitochondrial membranes or the electrode response.

The lipid peroxidation effect on ΔΨ induced by ADP/Fe²⁺ on respiring mitochondria was carried out as previously described for ΔΨ determinations, but the medium was supplemented with 1.5 μg of oligomycin per milligram of mitochondrial protein to prevent any depolarization associated with phosphorylation that will follow ADP/FeSO₄ addition.
Lipid Peroxidation Evaluation. Lipid peroxidation in rat liver mitochondria was measured as previously described by Sassa et al. (1990), by monitoring at 25°C the oxygen consumption with a Clark-type oxygen electrode in a total volume of 1 ml. The amount of mitochondria was 1 mg of protein and the incubation medium consisted of 175 mM KCl, 10 mM Tris-HCl, pH 7.4, supplemented with 3 μM rotenone. Peroxidation was started by simultaneously adding final concentrations of 1 mM ADP and 0.1 mM FeSO₄ after a 3-min incubation period. The amount of oxygen consumed during peroxidation was calculated assuming that the saturated concentration of O₂ at 25°C is 238 μM. Lipid peroxidation was also measured by determining the amount of lipid peroxides formed during incubation as the amount of thiobarbituric acid reactive substances (TBARS) formed according to Rohn et al. (1993), with some modifications. Mitochondrial protein (3 mg) was incubated, at 25°C, in 3 ml of a medium consisting of 175 mM KCl, 10 mM Tris, pH 7.4, supplemented with 3 μM rotenone. The drugs (carvedilol and BM-910228) were allowed to incubate for 3 min before membrane lipid peroxidation was started by adding simultaneously ADP/FeSO₄ (1 mM/0.1 mM). At selected time intervals, samples of 0.3 ml were taken and centrifuged at 15,000 g for 5 min. The supernatant was titrated by five successive additions of FCCP (up to 25 μM) was added to allow the rapid oxygen consumption phase in a concentration-dependent manner. The two compounds have similar inhibitory kinetics of lipid peroxidation; they suppress the rapid oxygen consumption phase in a dose-dependent manner. The two compounds have similar inhibiting kinetics of lipid peroxidation: they suppress the rapid oxygen consumption phase in a concentration-dependent manner but do not suppress the lag phase. Apparently, neither drug affects the formation of the initiating complexes; they do prevent, however, the formation of two-phase kinetics: first, a slow oxygen consumption lag phase lasting about 1 min, followed by a rapid oxygen consumption phase. This lag time can be correlated to the initiating phase of lipid peroxidation were it is generally believed that there is formation of an ADP perferryl ion complex (ADP-Fe³⁺-O₂ • → ADP-Fe⁴⁺-O₂²⁻) (Sassa et al., 1990). The superoxide radical formation accounts for the oxygen consumption (Sassa et al., 1990). These initiating complexes promote the abstraction of a hydrogen atom and the formation of a lipid radical that absorbs oxygen-forming lipoperoxides. The lipoperoxides are then broken down and reorganized as lipid radicals that enter a free radical chain reaction usually called propagation phase. The propagation phase accounts for the oxygen consumption on the second rapid oxygen consumption phase (Sassa et al., 1990).

As shown in Fig. 1 both carvedilol and BM-910228 inhibited ADP/Fe³⁺ -induced lipid peroxidation in liver mitochondria in a dose-dependent manner. The two compounds have similar inhibiting kinetics of lipid peroxidation: they suppress the rapid oxygen consumption phase in a concentration-dependent manner but do not suppress the lag phase. Apparently, neither drug affects the formation of the initiating complexes; they do prevent, however, the free radical chain reactions. At high enough concentrations (carvedilol, 40 μM; BM-910228, 1 μM) a complete lipid peroxidation inhibition is observed.

Results

Antiperoxidative Effects on Rat Liver Mitochondria. Figure 1 shows the effect of different concentrations of both carvedilol (A) and BM-910228 (B) on oxygen consumption due to lipid peroxidation initiated by ADP/Fe³⁺ in isolated nonenergized rat liver mitochondria. In the absence of both tested drugs and after the addition of ADP/Fe³⁺, oxygen consumption quickly started. It was possible to distinguish a two-phase kinetics: first, a slow oxygen consumption lag phase lasting about 1 min, followed by a rapid oxygen consumption phase. This lag time can be correlated to the initiating phase of lipid peroxidation were it is generally believed that there is formation of an ADP perferryl ion complex (ADP-Fe³⁺-O₂ • → ADP-Fe⁴⁺-O₂²⁻) (Sassa et al., 1990). The superoxide radical formation accounts for the slow oxygen consumption (Sassa et al., 1990). These initiating complexes promote the abstraction of a hydrogen atom and the formation of a lipid radical that absorbs oxygen-forming lipoperoxides. The lipoperoxides are then broken down and reorganized as lipid radicals that enter a free radical chain reaction usually called propagation phase. The propagation phase accounts for the oxygen consumption on the second rapid oxygen consumption phase (Sassa et al., 1990).

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The results shown on Fig. 2 were taken from Fig. 1 considering that in the absence of both compounds oxygen consumption stabilized within 6 min after addition of ADP/Fe$^{2+}$. Rat liver mitochondria (1 mg) were incubated in 1 ml of medium consisting of 175 mM KCl, 10 mM Tris (pH 7.4), at 25°C, supplemented with 3 μM rotenone (3 μM), for 3 min. Peroxidation started by adding ADP (1 mM) and FeSO$_4$ (0.1 mM). RLM, oxygen consumption in the absence of rat liver mitochondria. Numerical values near traces indicate concentrations of drugs in micromolar range. Traces are representative of six independent experiments.

The results shown on Fig. 2 were taken from Fig. 1 considering that in the absence of both compounds oxygen consumption stabilized within 6 min after addition of ADP/Fe$^{2+}$. The amount of oxygen consumed by lipid peroxidation in the presence of carvedilol and BM-910228 during 6 min was compared with the result obtained in the absence of both compounds. The concentrations of both compounds required for 50% inhibition (IC$_{50}$) were 10.9 μM for carvedilol and 0.33 μM for BM-910228. Under the same conditions the IC$_{50}$ value of Trolox C, a well-known antioxidant analog of α-tocopherol (Carini et al., 1990) is 18.8 μM. BM-910228 shows a very potent antiperoxidation effect, being more than 30 times that of carvedilol.

The quantitative evaluation in terms of TBARS formation induced by ADP/Fe$^{2+}$ was carried out to confirm the clear antioxidant effects of the test compounds observed by monitoring the oxygen consumption. Figure 3 shows that the MDA formation induced by ADP/Fe$^{2+}$ follows a kinetics similar to that observed for oxygen consumption. The MDA formation was inhibited in the same range of concentrations of carvedilol and BM-910228 observed to inhibit oxygen consumption. It is noteworthy that the IC$_{50}$ values were approximately independent of the method used, either the TBARS method or the oxygen consumption.

Lipid Peroxidation Effects on Mitochondrial ΔΨ. Mitochondrial functions depend strictly on the maintenance of the ΔΨ generated by respiration. Any condition responsible for a disruption on inner mitochondrial membrane will cause the collapse of ΔΨ. Because the ΔΨ is the main component of the Δp it is of interest to monitor the drop in ΔΨ that follows membrane disruption linked to lipid peroxidation induced by oxidative stress. In fact, previous reports have shown that various pro-oxidants, such as Fe$^{2+}$-ascorbate or organic peroxides, known to activate lipid peroxidation, cause a time dependent full of ΔΨ concomitant to the accumulation of malonic dialdehyde (Masini et al., 1985).

Figure 4 shows the development of ΔΨ after mitochondrial energization with succinate. In this experiment the energization of mitochondria was carried out in the presence of oligomycin, a specific inhibitor of mitochondrial ATP synthase, to avoid the membrane depolarization that follows ADP/Fe$^{2+}$ addition. Upon succinate energization and in the absence of ADP/Fe$^{2+}$, mitochondria built up and sustained a ΔΨ close to 210 mV for more than 25 min. After the addition of ADP/Fe$^{2+}$ a lag phase was observed that represents the time required to generate a sufficient amount of ROS responsible for the initiation of lipid peroxidation; the potential underwent a rapid decline after this lag phase. This decline of the ΔΨ is associated with the disruption of mitochondrial membrane promoted by lipid peroxidation. Carvedilol and BM-910228 exhibited a dose-dependent clear-cut protection against the collapse of ΔΨ promoted by ROS. It is important to note that the maximum protection of the drugs on ΔΨ was attained in the concentration range of 40 μM carvedilol and 1 μM BM-910228 where drug concentrations show maximum antioxidant capabilities as determined by the quantification of TBARS and the monitoring of oxygen consumption. The drugs’ stabilizing action on mitochondrial inner membrane will preserve the capability of mitochondria to proceed energy linked processes such as oxidative phosphorylation in noxious conditions such as those associated with oxidative stress. It can also be observed from Fig. 4 that carvedilol, as
opposed to its metabolite, in the concentration range showing antiperoxidation action, consistently decreased the mitochondrial DC upon succinate energization.

Effects of Carvedilol and BM-910228 on Mitochondrial Respiration and DC. To elucidate the compounds’ effects on rat liver mitochondrial energetic metabolism, we studied the respiration parameters as well as the DC of energized mitochondria. The effects of carvedilol and BM-910228 on the respiration rates measured during and after a phosphorylation pulse induced by the addition of ADP are reported in Table 1. The ADP/oxygen ratios and DC are also indicated in Table 1. As shown in Table 1, the presence of carvedilol in the incubation medium did not alter significantly either the rate of the ADP-stimulated respiration (state 3) or the resting respiration rate (state 4) at concentrations up to 40 μM. RCR is a measure of the dependence of the respiratory rate on ADP; it indicates the ratio between the oxygen consumption rate in the presence of added ADP (state 3) and the rate obtained after the phosphorylation pulse is complete (state 4). Due to the ineffectiveness of carvedilol (up to 40 μM) on state 3 and state 4 respiration, RCR values did not suffer any alterations. The efficiency of mitochondrial oxidative phosphorylation determined by the ADP/oxygen ratio, i.e., the ratio between the nanomoles of ADP added and the nanograms of atoms of oxygen consumed during the phosphorylation pulse did not suffer any alteration as well. At concentrations higher than 40 μM carvedilol, however, the state 4 was strongly stimulated in a concentration-dependent manner, whereas the RCR and ADP/oxygen values gradually decreased. The DC, which supplies energy for ADP phosphorylation, was gradually decreased although not significantly up to 40 μM carvedilol (dropped approximately 14 mV for 40 μM), but dropped abruptly from 213 to 170 mV for the concentration of 100 μM carvedilol.

In Table 1, the same parameters are reported for BM-910228. As opposed to carvedilol, BM-910228 did not significantly affect any of the parameters studied up to the concentration range shown to have maximal antioxidant effects (up to 1 μM). Therefore, mitochondrial energetics seem to be substantially unaffected by the metabolite of carvedilol.

Carvedilol seems not to impair mitochondrial oxidative phosphorylation at concentrations up to 40 μM, despite the
In vitro effects of carvedilol and BM-910228 on respiration and steady-state $\Delta \Psi$ of rat liver mitochondria

The values are means $\pm$ S.E.M. of six independent experiments. State 3 and state 4 respiration is rate of oxygen uptake (nmol of O$_2$/mg/min). $\Delta \Psi$ is the transmembrane electric potential upon succinate energization of mitochondria.

**TABLE 1**

<table>
<thead>
<tr>
<th>Carvedilol (µM)</th>
<th>State 4</th>
<th>State 3</th>
<th>RCR</th>
<th>ADP/Oxygen</th>
<th>$\Delta \Psi$</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>15.57 ± 2.05</td>
<td>65.70 ± 2.87</td>
<td>4.34 ± 0.42</td>
<td>2.01 ± 0.07</td>
<td>0.15 ± 4.1</td>
</tr>
<tr>
<td>5</td>
<td>15.82 ± 1.90</td>
<td>65.36 ± 3.53</td>
<td>4.30 ± 0.48</td>
<td>2.05 ± 0.05</td>
<td>N.D.</td>
</tr>
<tr>
<td>10</td>
<td>16.00 ± 1.73</td>
<td>65.62 ± 3.25</td>
<td>4.36 ± 0.40</td>
<td>1.97 ± 0.06</td>
<td>203.7 ± 4.4</td>
</tr>
<tr>
<td>20</td>
<td>16.34 ± 2.06</td>
<td>68.71 ± 1.47</td>
<td>4.45 ± 0.49</td>
<td>2.03 ± 0.08</td>
<td>200.8 ± 4.5</td>
</tr>
<tr>
<td>30</td>
<td>16.45 ± 1.63</td>
<td>71.72 ± 3.34</td>
<td>4.50 ± 0.57</td>
<td>1.97 ± 0.04</td>
<td>197.0 ± 4.7</td>
</tr>
<tr>
<td>40</td>
<td>18.06 ± 1.29</td>
<td>71.90 ± 3.69</td>
<td>4.24 ± 0.71</td>
<td>1.91 ± 0.15</td>
<td>194.4 ± 5.3</td>
</tr>
<tr>
<td>50</td>
<td>23.20 ± 1.65</td>
<td>72.31 ± 1.90</td>
<td>3.18 ± 0.31*</td>
<td>1.80 ± 0.13</td>
<td>N.D.</td>
</tr>
<tr>
<td>80</td>
<td>28.60 ± 3.20*</td>
<td>71.75 ± 1.60</td>
<td>2.63 ± 0.36**</td>
<td>1.46 ± 0.11**</td>
<td>N.D.</td>
</tr>
<tr>
<td>100</td>
<td>62.53 ± 4.03***</td>
<td>69.94 ± 1.67</td>
<td>1.14 ± 0.09***</td>
<td>1.29 ± 0.07***</td>
<td>171.3 ± 8.2</td>
</tr>
</tbody>
</table>

**BM910228 (µM)**

| 0              | 13.85 ± 1.94 | 69.92 ± 6.56 | 5.22 ± 0.44 | 1.93 ± 0.03 | 224.4 ± 0.5 |
| 0.12           | 12.99 ± 2.43 | 69.21 ± 6.25 | 5.45 ± 0.55 | 1.91 ± 0.07 | N.D. |
| 0.25           | 14.53 ± 2.55 | 72.31 ± 5.27 | 5.42 ± 0.78 | 1.92 ± 0.02 | 225.3 ± 2.4 |
| 0.5            | 14.36 ± 2.35 | 68.71 ± 4.75 | 5.12 ± 0.43 | 1.89 ± 0.05 | 222.8 ± 0.3 |
| 0.75           | 15.22 ± 2.96 | 71.45 ± 5.90 | 5.15 ± 0.70 | 1.88 ± 0.08 | 220.3 ± 1.3 |
| 1              | 13.98 ± 2.06 | 66.09 ± 4.17 | 5.21 ± 0.70 | 1.93 ± 0.06 | 219.6 ± 1.1 |

N.D., not determined.

* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Slight decrease of the $\Delta \Psi$ generated by mitochondrial respiration. To directly investigate carvedilol’s action sites responsible for this depressive effect on $\Delta \Psi$ we adopted the approach developed by Brand (1990).

**Top-Down Analysis of the Kinetic Responses of “Proton Leak”, “Substrate Oxidation”, and “Phosphorylation System” to Changes in $\Delta \Psi$ (Effects of Carvedilol on the Proton Leak through the Mitochondrial Membrane).** Putative proton leaks induced by carvedilol through the mitochondrial inner membrane were investigated in non-phosphorylating mitochondria titrated with malonate, a respiratory inhibitor, and $\Delta \Psi$ ($\Delta \Psi$ equals $\Delta \psi_0$ in the experimental conditions used) was plotted against respiration rate (Fig. 5A). In a steady state, the proton efflux must equal the proton leak, assuming that no slip in the proton pumps occurs. In addition, any secondary effect on the leak due to alterations in the $\Delta \psi$ value is eliminated. Therefore, if a given compound increases the proton leak across the mitochondrial inner membrane, the curve will be displaced downward and to the right. Carvedilol did increase the proton leak at the concentrations of 10 and 30 µM. The induction of proton leak was further confirmed by monitoring a slight increase of the mitochondrial swelling induced by carvedilol in iso-osmotic NH$_4$NO$_3$ (data not shown).

**Effects of Carvedilol on the $\Delta \psi$-Producing System.** Figure 5B shows the titration of nonphosphorylating mitochondria with an uncoupler (FCCP) to increase the rate of respiration and to decrease $\Delta \psi$. In this case, the plot obtained is a description of the kinetic response of the $\Delta \psi$ producers to their product, $\Delta \psi$. Consequently, when a compound inhibits any component of the $\Delta \psi$-producing system the curve will be displaced downwards and to the left. That is, at a given value of $\Delta \psi$ the respiration rate will decrease. In Fig. 5B, the presence of carvedilol displaces the titration of $\Delta \psi$ against respiration rate downwards and to the left. Therefore, carvedilol inhibits one or more of the components of the $\Delta \psi$-generating system (i.e., the electron transport system or the substrate transporters).

**Effects of Carvedilol on the Phosphorylation System.** Figure 5C shows the plot of $\Delta \psi$ against respiration rate of phosphorylating mitochondria titrated with a respiratory inhibitor. The respiration rate measured during the titrations is higher than that required to balance the influx of protons through the phosphorylation system (Brand, 1990). Therefore, any titration of phosphorylating mitochondria with a respiratory inhibitor produces a plot showing the kinetic dependence of the phosphorylation system on $\Delta \psi$. Because carvedilol was shown to induce proton leak, the respiration rates of Fig. 5C were corrected in accordance with the contribution of the respiration rates’ increase due to proton leak at given $\Delta \psi$ values (Brand, 1990). Apparently, carvedilol did not affect the phosphorylating system. To confirm the conclusion inferred from Fig. 5C we determined the effect of carvedilol on ATP synthesis by an alternative method because the correction procedure used in Fig. 5C is susceptible to experimental error. This was done by measuring the rate of ATP synthesis by means of a pH electrode, which follows the pH increase associated with the production of ATP, as a function of $\Delta \psi$. Figure 6 shows the rate of ATP synthesis as a function of $\Delta \psi$ and confirms the dependence of ATP synthesis on $\Delta \psi$ (Zoratti and Petronilli, 1985). From Fig. 6 it can be seen that ATP synthesis rates against $\Delta \psi$ are superimposable in the presence of carvedilol or control, which means that carvedilol does not affect the mitochondrial phosphorylation system for concentrations up to 40 µM. This conclusion is in accordance with the fact that carvedilol does not affect the ADP/oxygen ratio. It should be stressed that these conclusions are true for concentrations of carvedilol up to 40 µM, which were shown to have maximal antioxidant activity. Higher concentrations of carvedilol beginning at 50 µM (some variations are related to mitochondrial preparations) show toxic effects to mitochondria because they affect the phosphorylation efficiency on mitochondria (ADP/oxygen ratio is depressed).

Additional experiments were made to confirm that carvedilol does not affect directly mitochondrial ATP synthase (ATPase) activity. ATPase activity was determined using rat liver submitochondrial particles by monitoring the pH changes associated with ATP hydrolysis. Carvedilol showed no effect...
on ATPase activity using a carvedilol concentration of 40 \( \mu M \) with a protein concentration of 0.4 mg/ml (data not shown).

**Discussion**

Mitochondria are known to be very sensitive to oxidative stress conditions (Castilho et al., 1994), which explains the reported impairment of mitochondrial functions associated with some pathological states. Mitochondria have substantial capacity to generate ROS, especially after ischemia and reperfusion (Ambrosio et al., 1993). In vivo experiments have shown overproduction of ROS by mitochondria isolated from brain, heart, liver, and other organs as well as culture cells (Ambrosio et al., 1993; Lemasters and Nieminen, 1997). Moreover, in vitro experiments have shown that mitochondria isolated from pathological tissues such as tumor produced more ROS than those produced by normal mitochondria (Konstantinov et al., 1987).

To highlight the importance of carvedilol and its metabolite BM-910228 in the protection of mitochondria from oxidative stress conditions we studied their antioxidant capacity in a model of severe oxidative damage induced by ADP/FeSO\(_4\). Our study showed that both compounds have potent antioxidative effects on mitochondrial membranes with IC\(_{50}\) values very similar to those reported for brain homogenates (8.1 \( \mu M \) for carvedilol; 0.3 \( \mu M \) for BM-910228) in a Fe\(^{2+}\)-ascorbate-induced lipid peroxidation model (Yue et al., 1992). It is important to stress that propranolol, a \( \beta \)-blocker commonly used in cardiovascular therapy, did not show any antioxidative activity in mitochondrial membranes (data not shown).

ADP and Fe\(^{2+}\) were chosen as lipid peroxidation inducers because increased concentrations of these compounds were reported in several pathological states associated with oxidative stress conditions (Radi et al., 1997). The iron content of mitochondria not bound in heme and FeS centers is chelated by substances such as ADP, ATP, GTP, and citrate (Tangeras et al., 1980). Additionally, it has been proposed that low-molecular-weight iron complexes are involved in the mechanisms of postischemic oxidative damage and liver injuries associated with iron diseases (Bacon and Britton, 1990).

The peroxidation of mitochondrial membranes induced by ADP/Fe\(^{2+}\) displays a concentration-dependent inhibition by the compounds, both showing inhibitory effects greater than that of the antioxidant Trolox C (IC\(_{50}\) = 18.8 \( \mu M \)), a vitamin E analog, shown to attenuate myocardial injury during ischemia and reperfusion (Rubinstein et al., 1992). BM-910228 displayed an exceptional antioxidative action 33 times stronger than that of carvedilol (Fig. 2).

Mitochondrial membrane lipid peroxidation results in irreversible loss of mitochondrial functions such as mitochondrial respiration, oxidative phosphorylation, and ion transport (Bacon and Britton, 1990). Previous studies have also demonstrated that the preservation of mitochondrial \( \Delta \Psi \)
critical to maintaining cell integrity (Carini et al., 1992). In this regard, mitochondrial oxidative damage resulting from oxidative stress has been demonstrated to be associated with a decrease in mitochondrial ΔΨ and depletion of cellular ATP as well as with cell death (Kowaltowski and Vercesi, 1999). Carvedilol and its metabolite, by preventing the disruption of mitochondrial ΔΨ that follows peroxidative alterations, might protect mitochondrial function thus preventing irreversible cell injury in pathological situations associated to cellular oxidative stress.

As opposed to BM-910228, carvedilol caused an apparent decrease of the mitochondrial ΔΨ upon succinate energization, at the concentration range shown to inhibit lipid peroxidation (Fig. 4). Hence, we studied the immediate effects of the compounds on mitochondrial electron transfer reactions and oxidative phosphorylation. Mitochondrial respiration rates and ΔΨ values are shown in Table 1. Carvedilol and its metabolite were tested up to the concentration where lipid peroxidation was completely inhibited; this break point was found at 40 μM carvedilol and at 1 μM BM-910228. BM-910228 up to 1 μM did not affect mitochondrial functions; neither the respiratory parameters nor mitochondrial ΔΨ were affected by the drug. On the other hand, when carvedilol was present, mitochondria developed a consistently lower ΔΨ. This decrease of the ΔΨ was accompanied by the consistent stimulation of state 4 respiration. At a carvedilol concentration of up to 40 μM, however, the mitochondrial phosphorylation efficiency is not affected because the ADP/oxygen ratio remains essentially unchanged. At 40 μM carvedilol, the ADP/oxygen ratio decreased by approximately 5%, reflecting a very slight energy dissipation. A gradual impairment of the energetic parameters appeared as the carvedilol concentration increased beyond 40 μM. Between 40 and 100 μM, it is apparent a more pronounced decrease in the ΔΨ, stimulation of state 4 respiration, and a decrease of RCR and ADP/P ratio, which reflects an impairment of the phosphorylation efficiency of mitochondria.

The effect of carvedilol on mitochondrial energy-linked processes can be ascribed to an uncoupling effect on oxidative phosphorylation. This uncoupling effect of carvedilol might be linked to a protonophoretic action on mitochondrial membrane. A classical uncoupling action of carvedilol can be considered on the basis of the following observations: 1) state 4 respiration is stimulated; 2) mitochondrial ΔΨ of respiring mitochondria is depressed; 3) oligomycin inhibition of stimulated ADP respiration is reverted by carvedilol (data not shown); and 4) mitochondria suspended in isosomotic NH₄NO₃ swell, confirming the enhancement of proton permeability. As reported by Cheng et al. (1996), the pKₐ for carvedilol is 7.9. Therefore, in the used medium (pH 7.2), 83.4% of carvedilol should be in the protonated, charged form, thereby allowing the molecules to behave like protonophores by entering energized mitochondria along the electrical gradient. Moreover, carvedilol is a highly lipophilic compound with an apparent partition coefficient (Log D octanol/H₂O) of 3.4 (Yue et al., 1992). A protonophoretic mechanism for carvedilol fits well with its lipophilicity, which favors the presence of the molecules in the bulk phase of the membrane, allowing a quick proton crossing through the membrane. It cannot be excluded, however, that the uncoupling action of carvedilol might be due to, at high concentrations, a nonspecific increase of the permeability of the inner mitochondrial membrane to ions, thereby leading to dissipation of ΔΨ and stimulation of state 4 respiration.

High concentrations of carvedilol (>40 μM) unequivocally affect the oxidative phosphorylation confirmed by the decrease of ADP/oxygen ratio. At concentrations of carvedilol below 40 μM, the level of respiratory ΔΨ is consistently depressed although the phosphorylation capacity of mitochondria remains essentially unaltered. The understanding of the mechanism responsible for this depressive effect on ΔΨ is crucial. To gain insight into the mode of carvedilol's mechanisms of action on mitochondria and to determine more precisely carvedilol sites of action on oxidative phosphorylation we used the top-down approach developed by Brand (1990).

The Brand approach demonstrated that carvedilol has two separate effects on mitochondrial oxidative phosphorylation: 1) it increases the proton leak through the inner mitochondrial membrane, thus confirming the previous results reported in Table 1 (Fig. 5A); and 2) it inhibits respiration (Fig. 5B). Carvedilol has no effect on the mitochondrial phosphorylation system (Fig. 5C). From Fig. 6 it can be seen that ATP synthesis rates against ΔΨ are essentially superimposable in the presence of carvedilol or of the control, which corroborates the data shown in Fig. 5C and the lack of any effect of carvedilol on the mitochondrial phosphorylation system. The fact that the ADP/oxygen ratio is not altered by the concentrations of carvedilol tested favors the proposition that ATP synthase was not affected. Furthermore, ATPase activity of liver submitochondrial particles, as assessed by monitortization of the pH decrease linked to ATP hydrolysis, was not affected by carvedilol (20–40 μM), further confirming the nonadverse effects on mitochondrial ATPase (data not shown).

Carvedilol could increase the proton leak through the mitochondrial inner membrane by acting as a proton shuttle or by disrupting the structure of the membrane. In fact, it was shown by Cheng et al. (1996) that carvedilol behaves as a membrane "fluidizer", which may alter membrane structure thus increasing the membrane's proton conductance and enhancement of proton leak. In our laboratory we confirmed that carvedilol (20 μM) has a fluidizing effect on mitoplasts of liver mitochondria, by using 1,6-diphenyl-1,3,5-hexatriene as a fluorescent probe for membrane microviscosity (data not shown).

The inhibition of the Δp producers by carvedilol could result from an interaction with the dicarboxilate carrier or with any component of the electron transport chain. Carvedilol may inhibit respiration through specific interactions with the electron transport chain or through alterations in mitochondrial inner membrane that affect protein-lipid interactions. At the moment, it is not possible to distinguish between these two alternatives.

Recent evidence suggests that mild uncoupling of mitochondria (depression of ΔΨ) may be an effective mechanism to reduce mitochondrial ROS production without seriously compromising cellular energetics (Korshunov et al., 1997). Therefore, it is tempting to speculate that the slight depressive effect of carvedilol (at nontoxic concentrations of carvedilol, i.e., ≤40 μM) on mitochondrial ΔΨ might have per se a protective effect on the cell by reducing the mitochondrial production of ROS in pathological situations associated to
oxidative stress. We are now currently testing this hypothesis.

The central pathological role for mitochondria in pathophysiological conditions associated with an excessive production of ROS by the organelle is well documented. The antioxidant properties of carvedilol may contribute to prevent cell necrosis and organ dysfunctions, through the preservation of mitochondrial functions, and may explain the strikingly superior cardioprotective effects of carvedilol relative to other drugs. Additionally, its metabolite BM-910228 with a notable antioxidant activity may significantly contribute to the therapeutic effects of the carvedilol.

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