

Inhibition of heart mitochondrial lipid peroxidation by non-toxic concentrations of carvedilol and its analog BM-910228

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Received 19 August 1999; accepted 22 May 2000

Abstract

Carvedilol, a non-selective β -adrenoreceptor blocker, has been shown to possess a high degree of cardioprotection in experimental models of myocardial damage. Reactive oxygen species have been proposed to be implicated in such situations, and antioxidants have been demonstrated to provide partial protection to the reported damage. The purpose of our study was to investigate the antioxidant effect of carvedilol and its metabolite BM-910228 by measuring the extent of lipid peroxidation in a model of severe oxidative damage induced by ADP/FeSO₄ in isolated rat heart mitochondria. Carvedilol and BM-910228 inhibited the thiobarbituric acid-reactive substance formation and oxygen consumption associated with lipid peroxidation with IC₅₀ values of 6 and 0.22 μ M, respectively. Under the same conditions, the IC₅₀ values of α -tocopheryl succinate and Trolox were 125 and 31 μ M, respectively. As expected, the presence of carvedilol and BM-910228 preserved the structural and functional integrity of mitochondria under oxidative stress conditions for the same concentration range shown to inhibit lipid peroxidation, since they prevented the collapse of the mitochondrial membrane potential ($\Delta\Psi$) induced by ADP/FeSO₄ in respiring mitochondria. It should be stressed that neither carvedilol nor BM-910228 induced any toxic effect on mitochondrial function in the concentration range of the compounds that inhibits the peroxidation of mitochondrial membranes. In conclusion, the antioxidant properties of carvedilol may contribute to the cardioprotective effects of the compound, namely through the preservation of mitochondrial functions whose importance in myocardial dysfunction is clearly documented. Additionally, its hydroxylated analog BM-910220, with its notably superior antioxidant activity, may significantly contribute to the therapeutic effects of carvedilol. © 2001 Elsevier Science Inc. All rights reserved.

Keywords: Carvedilol; BM-910228; Heart mitochondria; Antioxidants; Lipid peroxidation; Oxidative stress; Reactive oxygen species

1. Introduction

Carvedilol (Kredex®, Coreg®, Dibloc®; 1-(9*H*-carbazol-4-yloxy)-3-[[2-(2-methoxyphenoxy)ethyl]amino]-2-propanol) (Fig. 1) is an antihypertensive agent with multiple pharmacological properties of non-selective β -adrenergic antagonism with vasodilating properties exerted primarily through selective α_1 -blockade [1]. Carvedilol also has potent antioxidant action [2–5] as a superoxide and hydroxyl radical scavenger [3,6], and its chain-breaking activity suggests that

it is an efficient and powerful biological antioxidant [4]. Recent studies have demonstrated a beneficial role of carvedilol in the management of controlled congestive heart failure as well as of ischemic symptoms associated with stable and unstable angina pectoris [1]. The mechanisms underlying the multiple cardioprotective effects of carvedilol are not fully understood. However, its antioxidant activity is most likely responsible for its well-documented superior cardioprotection relative to typical β -blockers [1,7].

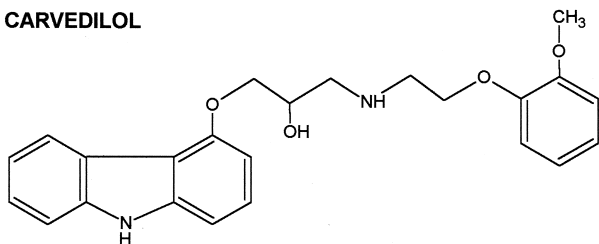
Substantial evidence supports the involvement of ROS in myocardial damage during ischemic insult [8] and especially during reperfusion that brings about additional damage [9–11]; these deleterious effects associated with ischemia/reperfusion conditions were shown to be minimized by antioxidant compounds [12]. The mitochondrial respiratory chain has long been recognized as a major endogenous source of ROS [13–15]. It has been estimated that during

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Abbreviations: TBARS, thiobarbituric acid-reactive substances; ROS, reactive oxygen species; TPP⁺, tetraphenylphosphonium ion; $\Delta\Psi$, mitochondrial membrane potential.

CARVEDILOL



BM 910228

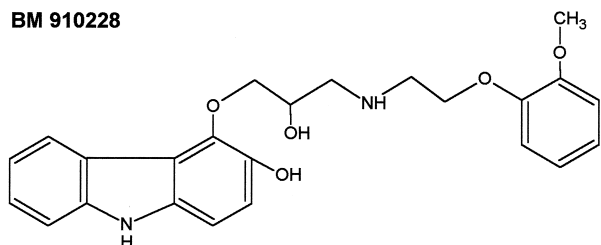


Fig. 1. Chemical structures of carvedilol 1-(9*H*-carbazol-4-yloxy)-3-[[2-(2-methoxyphenoxy)ethyl]amino]-2-propanol and BM-910228 (4-[[2-hydroxy-1-3-[[2-(2-methoxyphenoxy)ethyl]amino]propoxy]-9*H*-carbazol-3-ol]. BM-910228, one of the main metabolites of carvedilol, differs from carvedilol in being hydroxylated at position 3 of the carbazole group.

normal metabolism, 1–2% of oxygen reacting with the respiratory chain leads to the formation of superoxide radicals [16,17]. Under normal physiological conditions, mitochondrial antioxidant systems (enzymatic and non-enzymatic) scavenge free radicals and preserve mitochondrial integrity [18]; however, under certain pathophysiological conditions such as myocardial ischemia and especially during reperfusion [19,20], mitochondrial O_2^-/H_2O_2 generation dramatically increases [15,21]. The presence of polyunsaturated fatty acid-rich membranes enhances mitochondrial susceptibility to lipid peroxidation that can lead to membrane dysfunction and alterations in the structural and functional integrity of mitochondria, making these organelles potential contributors to ischemic and reperfusion injury [22,23]. In fact, previous studies showed that ROS are implicated in deleterious actions on mitochondrial structure and function that contribute to alterations of cardiac function [24]. Consequently, the antioxidant action of carvedilol and its hydroxylated metabolite BM-910228, which has been shown to possess superior antioxidant action compared to the parent compound [25], may contribute to the preservation of mitochondrial function, thus protecting myocardium from energy depletion under oxidative stress conditions.

The present study was undertaken to assess the *in vitro* antioxidant properties of carvedilol and its metabolite BM-910228 on lipid peroxidation initiated by ADP plus Fe^{2+} in isolated rat heart mitochondria. Additionally, the direct action of the compounds on mitochondrial bioenergetics was assessed for the purpose of clarifying whether the antioxidant doses of the compounds exhibit any mitochondrial toxicity so as to validate their therapeutic relevance.

2. Material and methods

2.1. Preparation of heart mitochondria

Heart mitochondria were prepared from adult Wistar strain rats (200–300 g) by conventional methods [26], with slight modifications. Rats were killed by cervical dislocation followed by decapitation, and the heart (1.0–1.5 g) was immediately excised and finely minced with a pair of sharp dissecting scissors in an ice-cold isolation medium containing 250 mM sucrose, 0.5 mM EGTA, 10 mM HEPES-KOH, pH 7.4. The minced blood-free tissue was then resuspended in 20 mL of isolation medium containing 0.1% (w/v) defatted bovine serum albumin (BSA; Sigma Chemical No. A-7030) and transferred to a 50-mL glass homogenizer vessel containing 20 mL of isolation medium supplemented with 0.5 mg of protease Type VIII (Sigma Chemical No. P-5390) per g of tissue and homogenized with a tightly fitted Potter–Elvehjen homogenizer. The suspension was incubated for 1 min (4°) and then rehomogenized. The homogenate was then centrifuged at 10,000 *g* for 10 min (Sorvall RC-5C, Plus, SS34 rotor, 4°). The supernatant fluid was decanted and the pellet, essentially devoid of protease, was gently homogenized to its original volume with a loose-fitting homogenizer. The suspension was centrifuged at 500 *g* for 10 min and the resulting supernatant was centrifuged at 10,000 *g* for 10 min. The pellet was resuspended using a paint brush and repelleted twice at 10,000 *g* for 10 min. EGTA and defatted BSA were omitted from the final washing medium. All mitochondrial isolation procedures were performed at 0–4°. For lipid peroxidation assays, the mitochondrial pellet was pelleted in a buffer consisting of 175 mM KCl, 10 mM Tris, pH 7.4, in order to remove sucrose (which may interfere with the thiobarbituric acid reaction). After isolation, the mitochondrial suspension was used after a 20-min recovery and within 4 hr. The isolation procedure yielded well-coupled mitochondria: the respiratory control ratio of the heart mitochondria varied from 8–10 (malate–glutamate as substrate) or 3–4 (succinate as substrate). Mitochondrial protein was determined by the biuret method [27], using BSA as standard.

2.2. Induction and assay of lipid peroxidation

Lipid peroxidation in rat heart mitochondria was measured as described by Sassa *et al.* [28] by monitoring, at 25°, the oxygen consumption with a Clark oxygen electrode of 1 mg mitochondria in a total volume of 1 mL of a medium consisting of 175 mM KCl, 10 mM Tris–HCl, pH 7.4, supplemented with 3 μ M rotenone. Peroxidation was started by adding final concentrations of 1 mM ADP and 0.1 mM $FeSO_4$ after a 2-min incubation period. The saturated concentration of O_2 in the incubation medium was assumed to be 238 μ M at 25°.

Lipid peroxidation was also measured by determining the amount of lipid peroxides formed during incubation as

the amount of TBARS formed according to Rohn *et al.* [29], with some modifications. Mitochondrial protein (3 mg) was incubated at 25° in 3 mL of a medium consisting of 175 mM KCl, 10 mM Tris, pH 7.4, supplemented with 3 μ M rotenone. The drugs (carvedilol and BM-910228) were allowed to incubate for 2 min before membrane lipid peroxidation was started by simultaneously adding ADP/FeSO₄ (1 mM/0.1 mM). At selected time intervals, samples of 0.3 mL were taken and mixed with 2.7 mL of a TBARS reagent consisting of 9% thiobarbituric acid (TBA), 0.6 N HCl, and 0.0056% 2,6-diterbutyl-4-methylphenol (BHT). The mixture was heated at 80–90° for 15 min and recooled in ice for 10 min before centrifugation in an Eppendorf centrifuge (1500 g, 5 min). Lipid peroxidation was estimated by the appearance of TBARS spectrophotometrically quantified at 535 nm. The amount of TBARS formed was calculated using a molar extinction coefficient of $1.56 \times 10^5 \text{ M}^{-1} \text{ cm}^{-1}$ and expressed as nmol TBARS/mg protein [30].

2.3. Oxygen consumption assays

Oxygen consumption was analyzed polarographically, at 25°, using a YSI Biological Oxygen Monitor (model 5300) and a Clark-type oxygen electrode (model 5331; Yellow Springs Instrument Co. Inc.) connected to a Kipp & Zonen chart recorder. Reactions were conducted in a 1-mL thermostatically closed and magnetically stirred glass vessel containing 1 mg of mitochondria in a respiration buffer of 130 mM sucrose, 50 mM KCl, 2.5 mM KH₂PO₄, 5 mM HEPES, pH 7.4, supplemented with 3 μ M rotenone. After a 2-min incubation period, mitochondrial respiration was initiated by addition of 8 mM succinate. Respiratory states were as defined by Chance and Williams [31]. State 3 rates were measured after the addition of 8 mM succinate and 0.25 mM ADP; state 4 rates were measured as the rates of oxygen consumption after exhaustion of ADP. Respiratory control ratios (RCR = state 3/state 4 rate), used to assess functional integrity, and ADP/O ratios were determined according to Estabrook [32]. The scale of oxygen uptake was calibrated according to the oxygen consumed by submitochondrial particles after the addition of titrated solutions of NADH.

In order to examine the effects of the drugs on mitochondrial respiration, we added the respiratory substrates after an incubation period of 2 min. Drugs were prepared in absolute ethanol. Concentration of ethanol in the assay medium was less than 0.2%, the concentration also used in the controls.

2.4. Mitochondrial membrane potential ($\Delta\Psi$) measurements

The mitochondrial membrane potential ($\Delta\Psi$) was estimated by continuously monitoring the accumulation and release of the lipophilic cation TPP⁺ with a TPP⁺-sensitive electrode prepared in our laboratory, according to Kamo *et al.* [33]. Therefore, the calculated $\Delta\Psi$ values were uncor-

rected for possible volume changes induced by the compounds tested. A mini-electrode was constructed with a poly(vinyl chloride) tube sealed with a poly(vinyl chloride)-based membrane containing the tetraphenylboron ion (TPB⁻) as a cation exchanger and filled with a 10 mM TPP⁺ solution. The reference electrode was an Ag/AgCl-saturated electrode (Tacussel, Model MI 402). Both the TPP⁺ and the reference electrode were inserted into the chamber used for determination of mitochondrial oxygen consumption and were connected to a pH meter (Radiometer, Model PHM 84). The signals were simultaneously fed to a dual-trace potentiometric recorder (Kipp & Zonen, Model BD 112). Mitochondria were incubated in the reaction medium containing 3 μ M TPP⁺. This TPP⁺ concentration was chosen to achieve high sensitivity in measurements and to avoid possible toxic effects on mitochondria. The transmembrane electric potential was estimated from the following equation (at 25°), assuming that the TPP⁺ distribution between mitochondria and the medium follows the Nernst equation, as previously described [33]:

$$\Delta\Psi \text{ (mV)} = 59 \log (v/V) - 59 \log (10^{\Delta E/59} - 1)$$

where v , V , and ΔE stand for mitochondrial volume, volume of the incubation medium, and the deflection of the electrode potential from the baseline, respectively. No correction was made for the “passive” binding contribution of TPP⁺ to the mitochondrial membranes, because the purpose of the experiments was to show relative changes in potential rather than absolute values. Drugs were added in ethanolic solutions to the reaction medium supplemented with mitochondria and allowed to incubate for 2 min before the addition of the respiratory substrate or ADP/Fe²⁺. Neither carvedilol nor its metabolite affected TPP⁺ binding to mitochondrial membranes or the electrode response. Calculations of the transmembrane potential were based on a matrix volume of 1.1 μ L/mg protein [34]. The lipid peroxidation effect on $\Delta\Psi$ induced by ADP/Fe²⁺ was carried out as previously described for $\Delta\Psi$ determination using the respiratory medium supplemented with 1.0 μ g oligomycin as reaction medium to prevent any depolarization associated with phosphorylation that might follow the addition of ADP/Fe²⁺.

2.5. Statistical analysis

Results are presented as means \pm SEM for the number of experiments indicated in the legends to the figures. Statistical evaluation was performed using the two-tailed Student's *t*-test; differences with a value of $P < 0.05$ were considered significant.

2.6. Calculation of IC_{50} values

The protective activity of the drugs tested was calculated as percent inhibition of TBARS formation obtained in the

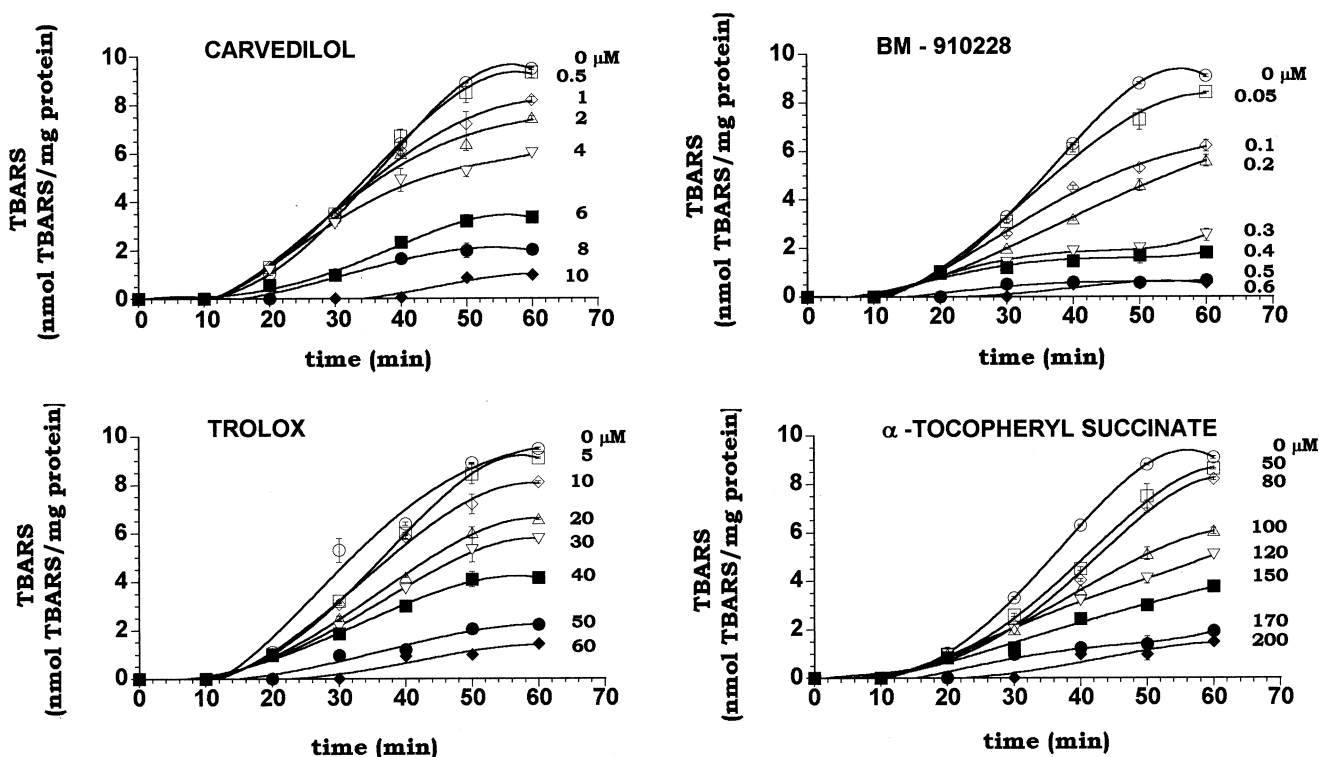


Fig. 2. Amounts of TBARS formed during lipid peroxidation in rat heart membranes induced by ADP/Fe²⁺ (1 mM/0.1 mM). Rat heart mitochondria (1 mg) were incubated in 1 mL of medium consisting of 175 mM KCl, 10 mM Tris (pH 7.4) supplemented with 3 μM rotenone at 25° for 2 min. Peroxidation was started by adding 1 mM ADP and 0.1 mM FeSO₄. Data are means \pm SEM of 4–5 experiments, each done in duplicate.

absence of the inhibitor (assumed to be 100%). We determined IC₅₀ values by using the GraphPad Prism program, version 2.0, distributed by GraphPad Software, Inc.

2.7. Chemicals

Carvedilol and BM-910228 were obtained from Boehringer Mannheim and used in ethanolic solutions. Other reagents were of the highest grade commercially available.

3. Results

In this study, we examined the antioxidant effect of carvedilol and its hydroxylated analog BM-910228 by measuring the extent of lipid peroxidation in a model of severe oxidative damage induced by ADP (1 mM)/FeSO₄ (0.1 mM) in isolated non-energized rat heart mitochondria. For comparison, we also examined the effects of the well-known antilipid peroxidation reagents α -tocopherol and Trolox [35]. First, we carried out the quantitative evaluation of their antiperoxidation effects on mitochondrial membranes in terms of TBARS formation induced by ADP/Fe²⁺ (Fig. 2). Clearly, carvedilol and BM-910228 dose-dependently inhibited ADP/Fe²⁺-induced lipid peroxidation in heart mitochondria. To confirm the clear antioxidant effects of the test compounds observed by use of the TBARS assay,

we monitored the time-dependent change in the peroxidation-related oxygen concentration of the rat heart mitochondrial suspension. As shown in Fig. 3, slow oxygen consumption was observed for a few minutes after the addition of ADP/Fe²⁺, after which the consumption of oxygen increased dramatically (Fig. 3, trace 0 μM). The time associated with the slow oxygen consumption that followed the addition of ADP/Fe²⁺ until the rapid oxygen uptake (lag time) is regarded to be the time required for the generation of a sufficient amount of ROS derived from ADP/Fe²⁺, such as the perferryl complex ADP-Fe³⁺-O₂⁻, responsible for the induction of lipid peroxidation [28,36,37]; the rapid oxygen consumption after this lag phase follows the peroxidative radical chain proliferation of membrane lipids by ROS. The extent of rapid oxygen consumption can be regarded as a good index of lipid peroxidation, seeing that it correlates well with the amount of TBARS formed from peroxide of fatty acid chains of mitochondrial phospholipids with TBARS [38].

The ability of the test compounds to inhibit lipid peroxidation in rat heart mitochondria was compared, as shown in Fig. 4. Carvedilol and BM-910228 dose-dependently inhibited ADP/Fe²⁺-induced lipid peroxidation. The concentration for 50% inhibition of the peroxidation (IC₅₀ values), as summarized in Fig. 4, decreased in the order BM-910228 > carvedilol > α -tocopheryl succinate > Trolox. The IC₅₀ values of carvedilol and BM-910228 were 6 and 0.22 μM ,

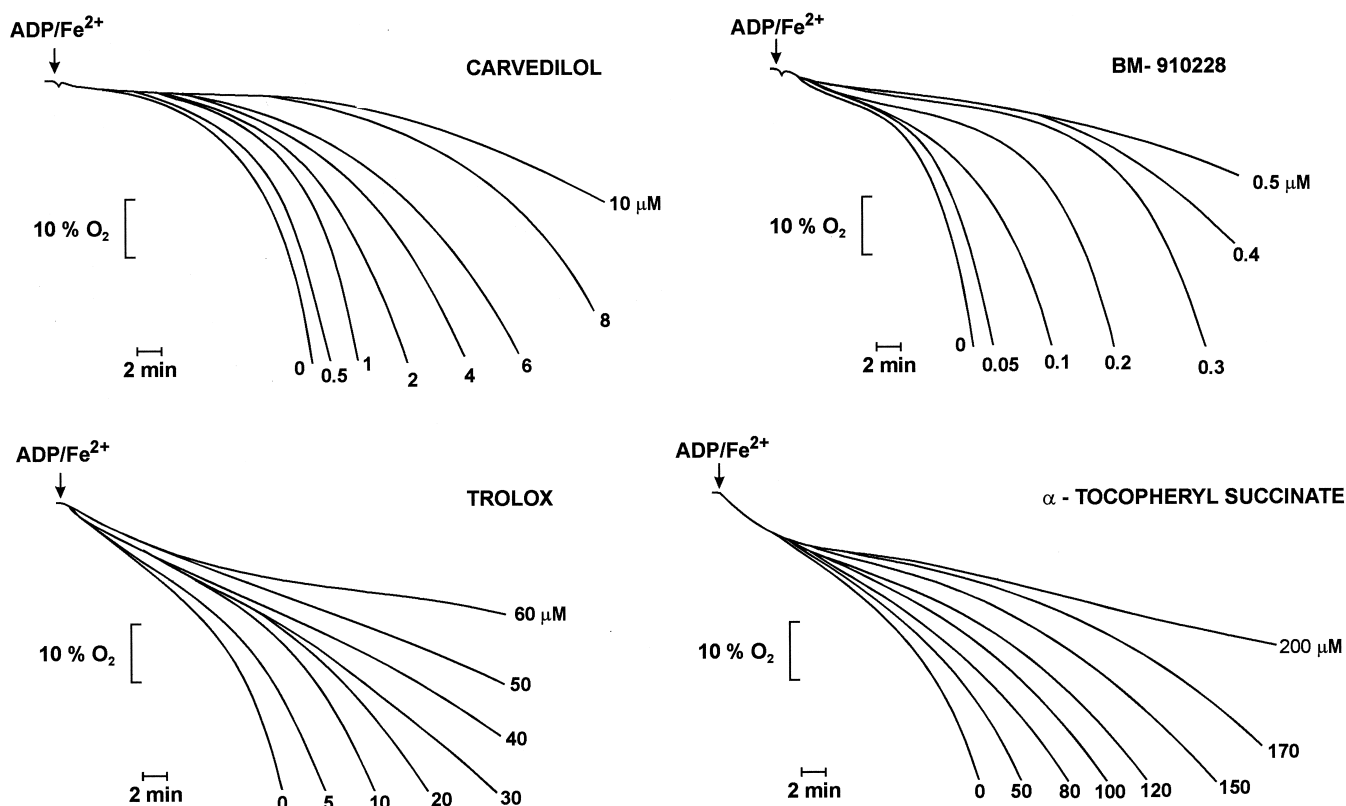


Fig. 3. Effects of carvedilol, BM-910228, Trolox, and α -tocopheryl succinate on O_2 consumption due to lipid peroxidation of isolated rat heart mitochondrial membranes induced by ADP and Fe^{2+} . Rat heart mitochondria (1 mg) were incubated in 1 mL of medium consisting of 175 mM KCl, 10 mM Tris (pH 7.4) at 25°, supplemented with 3 μ M rotenone. Peroxidation was started by adding 1 mM ADP and 0.1 mM $FeSO_4$. Numerical values adjacent to traces indicate concentrations of drugs in μ M. The traces represent typical recordings from several (4–5) experiments, each performed in duplicate.

respectively. Under the same conditions, the IC_{50} values of α -tocopheryl succinate and Trolox were 125 and 31 μ M, respectively. It is noteworthy that the IC_{50} values were the same independently of the method used, i.e. the TBARS

method or oxygen uptake. Carvedilol and especially BM-910228 were found to be much more potent antiperoxidation reagents than α -tocopheryl succinate and Trolox.

The energetic efficiency of the respiratory chain is clearly linked to the intactness of the inner mitochondrial membrane and to its impermeability to protons [38]. It is generally accepted that lipid peroxidation processes disturb mitochondrial energetic efficiency. In fact, it was demonstrated that lipid peroxidation of mitochondrial membranes correlates with changes in membrane integrity, causing their irreversible swelling, disruption, and the efflux of cations from the mitochondria [39]. The mitochondrial functions strictly depend on the maintenance of the proton motive force (Δp) generated by respiration. Any condition that brings about a disruption of the inner mitochondrial membrane will cause the collapse of Δp . Since $\Delta \Psi$ is the main component of the Δp , it was of interest to monitor the drop in $\Delta \Psi$ that follows membrane disruption linked to lipid peroxidation induced by oxidative stress. Fig. 5 shows the development of $\Delta \Psi$ 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, in an attempt to avoid the membrane depolarization that follows upon addi-

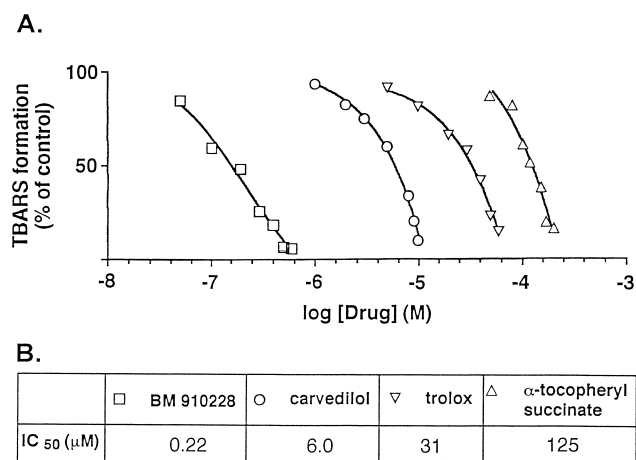


Fig. 4. (A) Concentration dependence of antiperoxidative activities of carvedilol, BM-910228, Trolox, and α -tocopheryl succinate in rat heart mitochondria. Antiperoxidative activity, shown as % control, was taken from the results of Fig. 2. (B) The IC_{50} values for the different compounds calculated from A.

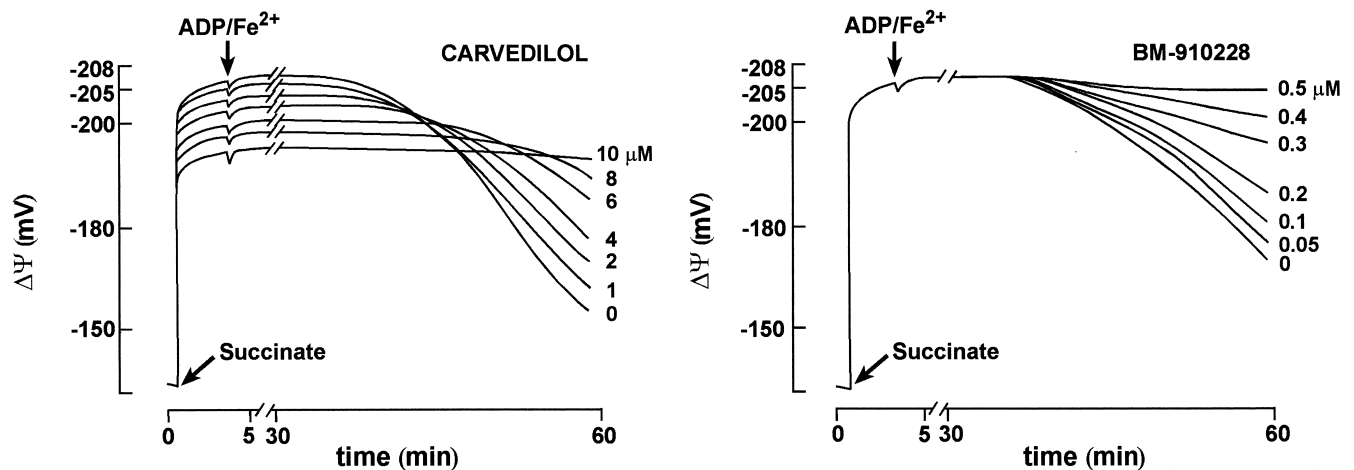


Fig. 5. Effect of carvedilol and BM-910228 on the mitochondrial transmembrane potential ($\Delta\Psi$) decrease induced by ADP and Fe^{2+} . Rat heart mitochondria (1 mg) were incubated in 1 mL of medium consisting of 130 mM sucrose, 50 mM KCl, 2.5 mM KH_2PO_4 , 5 mM HEPES (pH 7.4), at 25° supplemented with 1 μg oligomycin, 3 μM rotenone, and 3 μM TPP^+ . After succinate (8 mM) energization (2–3 min) in the absence (control) or presence of increasing concentrations of drugs, peroxidation was started by adding 1 mM ADP and 0.1 mM FeSO_4 . Concentration (in μM) of tested compounds is shown beside traces. The traces represent typical recordings from several (4–5) experiments, each performed in duplicate.

tion of ADP/Fe^{2+} . Fig. 5 shows that under succinate energization, mitochondria built up and sustained for more than 60 min, a $\Delta\Psi$ close to -205 mV when ADP/Fe^{2+} was absent. After the addition of ADP/Fe^{2+} , a lag phase followed that represents the time required for the generation of a sufficient amount of ROS responsible for the initiation of lipid peroxidation; the potential underwent a rapid decline after this lag phase. This decline in the $\Delta\Psi$ represents the disruption of mitochondrial membrane promoted by lipid peroxidation. Carvedilol and BM-910228 exhibited a dose-dependent, clear-cut protection against the collapse of $\Delta\Psi$ promoted by ROS. It is important to note that the maximum protection of the drugs afforded $\Delta\Psi$ was attained in the concentration range of 10 μM of carvedilol and 0.5 μM of BM-910228, i.e., the drug concentrations showing maximum antioxidant capabilities as determined by the quantification of TBARS and the monitoring of oxygen consumption. The stabilizing action of the drugs on mitochondrial inner membrane preserves the capability of mitochondria to

participate in energy-linked processes such as oxidative phosphorylation under noxious conditions such as those associated with oxidative stress.

It is noteworthy that carvedilol and BM-910228 did not affect mitochondrial function in the concentration range showing both electric potential preservation and antiperoxidation action. In fact, the drugs did not affect either the respiratory control ratio (RCR) or the phosphorylation index (ADP/O ratio) of heart mitochondria, suggesting a protective action on the structural and functional capability of mitochondria (Table 1).

In order to further confirm the non-toxic nature of carvedilol and BM-910228 on heart mitochondria, we measured the $\Delta\Psi$ fluctuations associated with mitochondrial respiration and the phosphorylation cycle induced by ADP. Fig. 6 shows that control mitochondria, upon the addition of succinate, developed a $\Delta\Psi$ close to -200 mV (state 4 condition). Addition of ADP caused an immediate fall to -170 mV (state 3 condition), a decrease that corresponds to the

Table 1

Effects of carvedilol and BM-910228 on heart mitochondrial respiratory parameters RCR (respiratory control ratio) and ADP/O

Carvedilol (μM)	Effect of carvedilol on		BM-910228 (μM)	Effect of BM-910228 on	
	RCR	ADP/O		RCR	ADP/O
0.0	3.23 \pm 0.05	1.55 \pm 0.04	0.0	3.23 \pm 0.05	1.55 \pm 0.04
1.0	3.28 \pm 0.10	1.58 \pm 0.05	0.05	3.10 \pm 0.08	1.58 \pm 0.04
2.0	3.20 \pm 0.14	1.61 \pm 0.04	0.1	3.10 \pm 0.08	1.59 \pm 0.03
4.0	3.30 \pm 0.25	1.59 \pm 0.04	0.2	3.15 \pm 0.11	1.59 \pm 0.04
6.0	3.38 \pm 0.15	1.60 \pm 0.02	0.3	3.13 \pm 0.10	1.59 \pm 0.02
8.0	3.13 \pm 0.19	1.60 \pm 0.03	0.4	3.18 \pm 0.01	1.61 \pm 0.03
10.0	3.13 \pm 0.05	1.61 \pm 0.05	0.5	3.20 \pm 0.08	1.60 \pm 0.03

Rat heart mitochondria (1 mg) incubated in 1 mL of medium consisting of 130 mM sucrose, 50 mM KCl, 2.5 mM KH_2PO_4 , 5 mM HEPES (pH 7.4), at 25°, supplemented with 3 μM rotenone, were energized with 8 mM succinate to induce state 4 respiration. State 3 respiration was initiated by adding 0.25 mM ADP. Data are means \pm SEM of 4–5 experiments, each performed in duplicate.

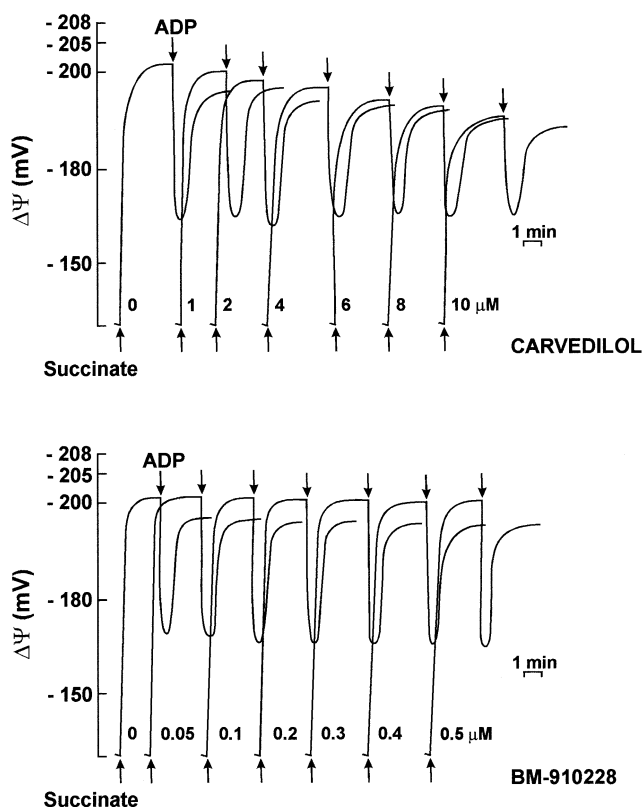


Fig. 6. Effects of carvedilol and BM-910228 on mitochondrial transmembrane potential fluctuations associated with respiration and the phosphorylative cycle. Rat heart mitochondria (1 mg) in 1 mL of medium consisting of 130 mM sucrose, 50 mM KCl, 2.5 mM KH_2PO_4 , 5 mM HEPES (pH 7.4), at 25°, supplemented with 3 μM rotenone and 3 μM TPP^+ , were energized with 8 mM succinate after a 2-min incubation. Addition of 0.25 mM ADP induced the state 3 condition (depolarization) followed by repolarization. Concentrations (in μM) of tested compounds are indicated on traces. The traces represent typical recordings from several (4–5) experiments, each performed in duplicate.

energy utilized for ATP synthesis. When the phosphorylation cycle was completed (after a lag phase of about 1 min), the $\Delta\Psi$ almost returned to its initial value. BM-910228, in the concentration range 0.1–0.5 μM , did not affect either the energization by succinate or the $\Delta\Psi$ fluctuations linked to the phosphorylation cycle, confirming that the drug does not affect either the respiratory chain or the phosphorylation system of mitochondria. Higher concentrations of BM-910228, in the range of 10 μM , did not affect mitochondrial function (data not shown). As opposed to BM-910228, carvedilol, although it did not affect the $\Delta\Psi$ fluctuations associated with the phosphorylation cycle, did show a slight depressive effect on the $\Delta\Psi$ generated by mitochondrial respiration; carvedilol consistently decreased the $\Delta\Psi$, which declined only about 8 mV for the maximum concentration of carvedilol used (10 μM). Furthermore, the depolarization induced by ADP in the presence of 10 μM carvedilol was only 25 mV compared to the value of 34 mV in the control. Nevertheless, carvedilol did not alter the phosphorylation efficiency of heart mitochondria, since the

ADP/O ratios were not affected by the presence of the compound.

The non-toxic effect of carvedilol on mitochondria was further confirmed by monitoring the pH changes associated with mitochondrial ATP synthesis. Carvedilol even at higher concentrations (up to 20 μM), much higher than those shown to inhibit mitochondrial lipid peroxidation, failed to induce adverse effects on mitochondrial ATP synthase, since both the rate and amplitude of pH changes linked to ATP synthesis were not affected (data not shown). Furthermore, ATPase activity of bovine heart submitochondrial particles, as assessed by monitoring the pH decrease linked to ATP hydrolysis, was not affected by high concentrations of carvedilol (up to 40 μM), further confirming the non-adverse effects on mitochondrial ATP synthase/ATPase (data not shown).

4. Discussion

It is well known that mitochondria are very sensitive to oxidative stress conditions [40], which explains the reported impairment of mitochondrial functions associated with pathological conditions such as ischemia and reperfusion [41,42]. To highlight the importance of carvedilol and its metabolite BM-910228 in the protection of mitochondria from oxidative stress conditions, we examined their effect on ADP/ Fe^{2+} -induced lipid peroxidation of rat heart mitochondrial membranes. With this system, active oxygen species chelated with Fe^{3+} and ADP such as the ADP- Fe^{3+} - O_2^- complex are thought to be initiators of radical chain reactions [43].

Our study showed that both carvedilol and BM-910228 have potent antiperoxidative effects on heart mitochondrial membranes. The IC_{50} values for carvedilol (6 μM) and BM-910228 (0.22 μM) found for rat heart mitochondrial membranes (Fig. 4) are very similar to those previously reported for brain homogenates of 8.1 μM (carvedilol) and 0.3 μM (BM-910228) in a Fe^{2+} -ascorbate-induced lipid peroxidation model [6]. In our studies, ADP and Fe were chosen as lipid peroxidation inducers, because increased concentrations of these compounds were reported in ischemia and postischemia reperfusion situations [18].

Our results clearly demonstrate that the peroxidation of mitochondrial membrane lipids induced by ADP/ Fe^{2+} displays a concentration-dependent inhibition by carvedilol and BM-910228. The antiperoxidative effects of carvedilol and BM-910228 were different, with a complete peroxidative inhibition at 10- and 0.5- μM concentrations, respectively. The inhibitory effects of carvedilol and BM-910228 were much greater than those of α -tocopheryl succinate (IC_{50} = 125 μM) and Trolox C (IC_{50} = 31 μM). Previous studies have demonstrated that α -tocopherol and Trolox, a water-soluble analog of α -tocopherol, may attenuate myocardial injury from ischemia and reperfusion [44]. It is

noteworthy that, in our study, carvedilol showed an antioxidant activity in rat heart mitochondria 20-fold greater than that of α -tocopheryl succinate, while its metabolite BM-910228 displayed an exceptional antiperoxidative action 27 times stronger than that of carvedilol (Fig. 4).

The evaluation of mitochondrial transmembrane electric potential ($\Delta\Psi$) is most important in studies of mitochondrial functions, since it represents the main component of the electrochemical gradient generated by mitochondrial respiration and accounts for more than 90% of the total available energy [45]. The energetic efficiency of mitochondria is clearly linked to the intactness of the inner mitochondrial membrane. It has been shown that lipid peroxidation processes correlate with alterations in the integrity of mitochondrial membranes, which cause perturbation of mitochondrial functions [46]. As expected, the induction of lipid peroxidation with ADP/Fe²⁺ brought about a drop in the $\Delta\Psi$ generated by mitochondrial respiration. Carvedilol and BM-910228 suppressed the de-energization of mitochondria at the concentration range shown to inhibit mitochondrial membrane lipid peroxidation. These data clearly indicate that carvedilol and its metabolite may contribute to the preservation of mitochondrial function in pathological situations associated with cellular oxidative stress.

It is of utmost importance that neither carvedilol nor BM-910228 induced any toxic effects on mitochondrial function, since mitochondrial phosphorylation efficiency is not affected by the concentration range of the compounds that inhibit the peroxidation of mitochondrial membranes, as was shown by the lack of significant alterations in the respiratory control index and the ADP/O ratios of mitochondria. To confirm the non-toxic nature of the compounds, we further evaluated the $\Delta\Psi$ fluctuations associated with mitochondrial energization and the phosphorylation cycle. Indeed, the results show that there were no significant alterations in the profile of $\Delta\Psi$ fluctuations, with a single exception: carvedilol slightly, but consistently, depressed the $\Delta\Psi$ generated by respiration. This is a striking observation in view of the fact that the compound does not compromise the mitochondrial phosphorylation capacity. Carvedilol's depressive effect might be related to the induction of the permeabilization of the inner mitochondrial membrane to protons. This was confirmed using the top-down approach of Brand [47]; non-phosphorylating mitochondria were titrated with malonate (an inhibitor of the respiratory chain) and the respiratory activities and corresponding $\Delta\Psi$ changes were determined simultaneously (data not shown). We concluded that carvedilol induces a slight membranar proton leak for the concentration range used (up to 10 μ M). This effect may be related to a mild protonophoretic action of the compound. Recent evidence suggests that mild uncoupling of mitochondria (depression of $\Delta\Psi$) may be an effective mechanism to reduce mitochondrial ROS without seriously compromising cellular energetics [48]. Therefore, the slight depressive effect of carvedilol on mitochondrial $\Delta\Psi$ might have a protective effect per se

on the cell. In fact, it was shown previously that mitochondria greatly reduce the production of ROS when the $\Delta\Psi$ is only slightly decreased by using very low concentrations of a protonophore [49]. Thus, this mechanism may be added to other known effective protective mechanisms of carvedilol, such as superoxide and hydroxyl scavenger activity and chain-breaking activity [2–6].

The aim of our investigation was to study the possibility of a direct protective effect of carvedilol and its metabolite on isolated heart mitochondria under oxidative stress conditions. Our results indicate that relatively low concentrations of carvedilol and BM-910228 protect mitochondria from the deleterious action of membrane lipid peroxidation. Most importantly, we also show that both compounds in the concentration range showing their antioxidant action do not affect mitochondrial bioenergetics.

Lipid peroxidation inhibition achieved *in vitro* by carvedilol occurs at micromolar levels, whereas the plasma peak concentration of carvedilol in humans has been reported to be 0.3 μ M after an oral dose of 50 mg [50]. Therefore, the results reported in our study would be relevant only if some type of concentration process exists. In fact, carvedilol is a highly lipophilic compound with a partition coefficient (log P octanol-H₂O) of 3.4, which is similar to that of propranolol [6]. It is meaningful that the accumulation of propranolol in Purkinje fibers and platelets reached concentrations up to 30- to 40-fold higher than plasma concentration [51]. Although we do not know the exact levels of carvedilol in lipid membranes, the large distribution volume of carvedilol (132 L) in humans indicates that the drug is extensively distributed to the tissue [52]. Therefore, it is quite conceivable that an effective level of carvedilol capable of inhibiting lipid peroxidation may be attained *in vivo*, which gives clinical relevance to carvedilol's antioxidant effect.

In conclusion, the antioxidant properties of carvedilol may contribute to the cardioprotective effect of the compound, namely through the preservation of mitochondrial functions whose importance in the pathogenesis of myocardial ischemia/reperfusion injury is clearly documented. Additionally, its hydroxylated analog BM-910228, one of the most important metabolites found in humans and one possessing superior antioxidant activity, may significantly contribute to the therapeutic effects of carvedilol.

Acknowledgments

This work was supported in part by grants from the Fundação para a Ciência e Tecnologia (FCT) (Project no. PRAXIS/PSAU/SAU/S/16/96) and from the Luso-Fármaco Company. We are grateful to Prof. G. Spöner of Boehringer Mannheim Pharmaceuticals for kindly providing us with BM-910228 and to Dr. Lino Gonçalves (Cardiology Service, University of Coimbra) for providing us with carvedilol.

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