

Advantages in the Use of Carvedilol versus Propranolol for the Protection of Cardiac Mitochondrial Function [84]

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ABSTRACT

Background: Carvedilol is a neurohormonal antagonist of multiple action which is used in clinical practice for the treatment of congestive heart failure, mild to moderate hypertension and myocardial infarction.

Previous results from our group have demonstrated that one of the main targets for the protective effect of carvedilol is the cardiac mitochondrial network. In this work, we compare the effect of carvedilol with propranolol in different models of mitochondrial dysfunction and in the generation of transmembrane electric potential (EP). We further tested if carvedilol was able to inhibit the mitochondrial permeability transition (MPT) induced by doxorubicin and calcium-dependent cytochrome *c* release, a phenomenon frequently associated with apoptotic cell death.

Methods: Cardiac mitochondria were isolated by differential centrifugation. Oxygen consumption and mitochondrial EP were determined using an oxygen electrode and a tetraphenylphosphonium-sensitive electrode, respectively. Changes in mitochondrial volume and the release of cytochrome *c* were measured with spectrophotometric techniques.

Results: Propranolol, compared with carvedilol, had only a marginal effect, not only in protection against MPT induction, but also against oxygen consumption linked to the oxidation of external NADH, a process that is considered by several authors as key in the

RESUMO

Vantagens na Utilização do Carvedilol em Relação ao Propranolol na Protecção da Função Mitochondrial Cardíaca

Introdução: O Carvedilol (CV) é um antagonista neuro-hormonal de múltipla acção que é usado na prática clínica para o tratamento da doença cardíaca congestiva, hipertensão ligeira a moderada e enfarte do miocárdio. Resultados anteriores do nosso grupo mostraram que um dos principais alvos para o efeito positivo do CV no miocárdio é a mitocôndria cardíaca. Neste trabalho, comparámos o efeito do CV com o propranolol em diferentes modelos de disfunção mitocondrial e na geração do potencial eléctrico transmembranar (PE). Averiguámos, também, se o carvedilol inibia a transição de permeabilidade mitocondrial (TPM) induzida por adriamicina e a libertação de citocromo *c* induzida por cálcio, um fenómeno frequentemente associado à morte celular por apoptose.

Métodos: Mitocôndrias cardíacas foram isoladas por centrifugação diferencial. O consumo de oxigénio e o PE mitocondrial foram determinados recorrendo a um eléctrodo de oxigénio e a um eléctrodo sensível a tetrafenilfosfónio, respectivamente. Variações de volume mitocondriais e a libertação de citocromo *c* mitocondrial foram determinados recorrendo a técnicas espectrofotométricas.

Resultados: O propranolol, quando comparado com o CV, teve um efeito marginal, não só a

cardiotoxicity of doxorubicin. Regarding EP generation, propranolol had no effect, in contrast to carvedilol, which was confirmed to act as a protonophore. For the first time we also show that carvedilol inhibits the MPT induced by doxorubicin and calcium-dependent cytochrome *c* release.

Conclusions: With this work, we further support the notion that carvedilol is effective in several models of mitochondrial dysfunction, particularly those involving oxidative stress. The results demonstrate that for some pathological conditions, carvedilol and propranolol have different mechanisms of action at the sub-cellular level, as propranolol seems to lack effectiveness in the protection of cardiac mitochondria.

Key words

Rat heart mitochondria; Carvedilol; Propranolol; Permeability transition; Doxorubicin

nível de protecção contra a TPM mas também ao nível da inibição da oxidação de NADH exógeno pelo Complexo I de mitocôndrias cardíacas isoladas, um processo associado por vários autores à cardiotoxicidade da adriamicina. Ao nível do PE mitocondrial, o propranolol mostrou não ter qualquer efeito visível, ao contrário do CV, que mostrou já possuir actividade protonofóretica. Pela primeira vez mostramos também uma inibição pelo CV da TPM induzida por adriamicina e a libertação e citocromo *c* induzida por cálcio.

Conclusões: Com este trabalho mostramos mais argumentos que demonstram que o CV é efectivo em vários modelos de disfunção mitocondrial, nomeadamente naqueles em que o dano mitocondrial é de natureza oxidativa. Os resultados demonstram que, para certas condições patológicas, o CV e o propranolol têm mecanismos diferentes de acção ao nível subcelular, sendo que o propranolol parece não ter efeitos protectores directos na mitocôndria cardíaca.

Palavras-Chave

Mitocôndria cardíaca; Carvedilol; Propranolol; Transição de permeabilidade; Adriamicina

INTRODUCTION

Carvedilol ([1-[carbazolyl-(4-oxy)]-3-[2-methoxyphenoxyethyl] amino]-propanol-(2)) is a multiple-action neurohormonal antagonist that is used clinically for the treatment of congestive heart failure, mild to moderate hypertension and myocardial infarction^(1, 2). Carvedilol competitively blocks 1, 2 and 1 adrenergic receptors, and possesses vasodilating properties that contribute to an overall cardiac sparing effect. Carvedilol also has potent antioxidant activity, responsible for an increased cardioprotection not shared by other β -blockers^(3, 4).

We have shown previously that carvedilol afforded antioxidant protection against cardiac mitochondria, particularly against peroxidation of mitochondrial membrane lipids⁽⁵⁾ and generation of reactive oxygen species (ROS) by the hypoxanthine / xanthine oxidase system⁽⁶⁾. Furthermore, we have demonstrated previously in isolated heart mitochondria that carvedilol was able to inhibit mitochondrial respiratory activity associated with complex-I-mediated external

NADH oxidation⁽⁷⁾. This oxidation was previously proposed to be due to an organoselective mitochondrial external NADH dehydrogenase^(8, 9), an enzyme responsible for the activation of doxorubicin, an antineoplastic agent, to a highly reactive semi-quinone form⁽¹⁰⁾. Further evidence for the antioxidant effect of carvedilol in heart mitochondria was the inhibition of the calcium-induced mitochondrial permeability transition (MPT)⁽¹¹⁾. This mitochondrial event takes place during mitochondrial calcium overload that may lead to enhanced ROS generation inside the mitochondrial matrix⁽¹²⁾. In addition, basic bioenergetic studies have shown evidence that carvedilol behaves as a weak protonophore in isolated heart mitochondria, thus decreasing the mitochondrial electric potential by several millivolts^(13, 14). We proposed that this factor could be advantageous in reducing the amount of ROS produced by the respiratory chain⁽¹⁴⁾, an effect that was demonstrated elsewhere by using small amounts of classic protonophores⁽¹⁵⁾.

The objective of this work was to demonstrate that some of the direct effects we have ob-

served so far in isolated heart mitochondria were specific to carvedilol and not shared by propranolol, a classic β -adrenergic receptor antagonist, also reported previously to possess antioxidant activity⁽¹⁶⁾. After comparing the protection of carvedilol and propranolol against calcium-induced MPT, we decided to use carvedilol alone against two models of oxidative stress-related mitochondrial dysfunction: calcium-induced cytochrome *c* release⁽¹⁷⁾ and the MPT induced by doxorubicin, calcium and exogenous NADH.

Our results showed that effects were specific for carvedilol and that this compound not only inhibited calcium-induced cytochrome *c* release, but also protected against doxorubicin-induced MPT. The results obtained show that a clinically significant difference between carvedilol and propranolol would be their differing direct impact on mitochondrial dysfunction.

METHODS

Materials. Carvedilol and BM-910228 were obtained from Roche Portugal (Lisbon) and prepared in dimethylsulfoxide (DMSO). All other compounds were purchased from Sigma Chemical Co. (St. Louis, MO).

Isolation of mitochondria from rat heart. Rat heart mitochondria from male Wistar rats (250-300 g) were prepared using a conventional procedure, as previously described⁽⁷⁾. Mitochondrial protein content was determined by the biuret method calibrated with BSA.

Measurement of mitochondrial transmembrane potential. The mitochondrial transmembrane potential ($\Delta\Psi$) was estimated with a TPP⁺ electrode as described by Kamo et al.⁽¹⁸⁾ without correction for the "passive" binding contribution of TPP⁺ to the mitochondrial membranes, since the purpose of the experiments was to show relative changes in potential rather than absolute values. A matrix volume of 1.1/ μ l/mg protein was assumed. Reactions were carried out, at 25 °C, in 2 ml of standard respiratory medium (100 mM KCl, 50 mM sucrose, 10 mM TRIS-MOPS, 30 μ M EGTA, 1 mM KH₂PO₄, pH 7.4) supplemented with 3 μ M TPP⁺ and 1 mg of mitochondria. Energized mitochondria were obtained with 8 mM succinate (plus μ 4 M rotenone) or 8 mM glutamate-malate.

Mitochondrial oxygen consumption sustained by exogenous NADH. The oxygen consumption

of isolated heart mitochondria was monitored polarographically with a Clark oxygen electrode connected to a suitable recorder in a 1-ml temperature-controlled water-jacketed closed chamber with magnetic stirring, at 25 °C. The standard respiratory medium consisted of 100 mM KCl, 50 mM sucrose, 10 mM TRIS-MOPS, 30 μ M EGTA, 1 mM KH₂PO₄, pH 7.4. 10 mM of NADH was added to induce mitochondrial respiration. When used, all compounds (100 μ M carvedilol, 100 μ M BM-910228 and 100 μ M propranolol) were pre-incubated with mitochondria at 25 °C for 3 minutes. All compounds were used in the same concentration used previously with carvedilol⁽⁷⁾ to show maximal effect. The mitochondria were suspended at a concentration of 1 mg/ml in the respiratory medium.

MPTP-related mitochondrial swelling. We followed changes in mitochondrial volume by monitoring the classic decrease in optical density at 540 nm related to MPT induction with a Jasco V-560 spectrophotometer. The assays were performed in 2 ml of reaction buffer with 200 mM sucrose, 10 mM TRIS-MOPS, 10 μ M EGTA, 5 mM KH₂PO₄, pH 7.4 and with 4 μ M rotenone, 8 mM succinate and 1 mg mitochondrial protein. The compounds were pre-incubated with the mitochondrial preparation for 3 minutes at 25 °C. The MPT was induced using 500 μ M CaCl₂. To assess doxorubicin-induced MPT induction, the assays were performed in 2 ml of the reaction media (200 mM sucrose, 10 mM TRIS-MOPS, 10 μ M EGTA, 5 mM KH₂PO₄, and supplemented with 4 M rotenone, 500 μ M NADH, 8 mM succinate and 0.5 μ g oligomycin). The mitochondria (0.5 mg) were incubated for three minutes with doxorubicin before addition of calcium.

Quantification of cytochrome *c* release: To quantify cytochrome *c* released on MPT induction, we used the spectrophotometric technique described by Appaix et al.⁽¹⁹⁾. Briefly, 4.2 mg of mitochondrial protein were incubated in 2 ml of reaction buffer (200 mM sucrose, 10 mM TRIS-KOH, 10 μ M EGTA and 5 mM KH₂PO₄, pH 7.4), supplemented with 12 μ M rotenone and 20 mM succinate. As a positive control, we used a hypo-osmotic buffer (60 mM KCl). Carvedilol (60 μ M) and cyclosporin-A (3 μ M) were pre-incubated with the mitochondrial suspension for 3 minutes before addition of calcium (500 nmoles / mg. mitochondrial protein). The

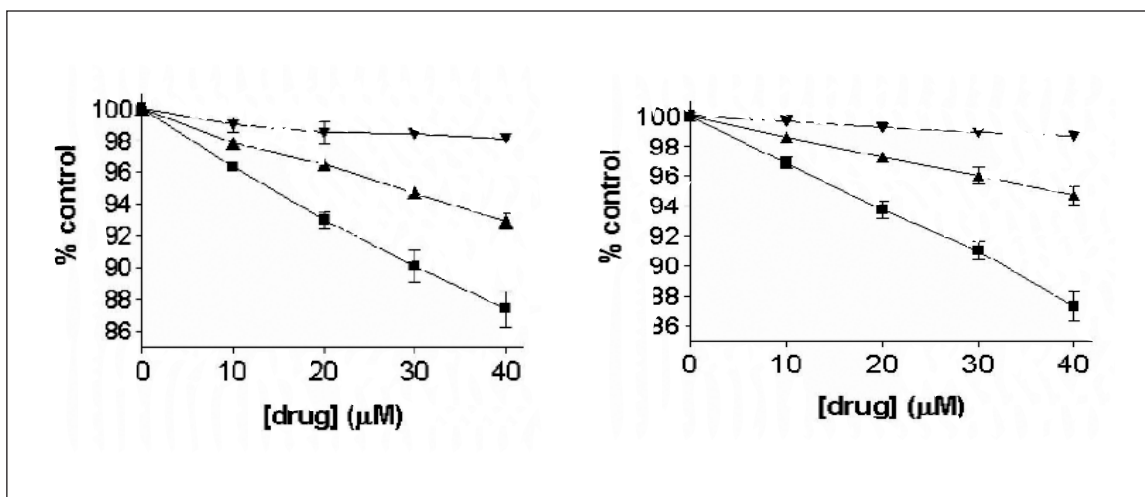


Fig. 1 Effects of carvedilol (square), BM-910228 (triangle) and propranolol (inverted triangle) on mitochondrial $\Delta\Psi$ generated by succinate (left) and glutamate-malate (right) in isolated heart mitochondria. Data are means \pm SEM of five different mitochondrial preparations.

samples were incubated at 25 °C for 20 minutes under constant stirring. The samples were then centrifuged and the supernatant filtered with a Millipore 0.2 filter, and the absorbance at 414 nm was read in a Jasco V-560 spectrophotometer. The values were obtained after a calibration curve made with known quantities of cytochrome *c*.

Statistical analyses – Analyses were conducted using the Student's *t* test for two groups or ANOVA followed by the Student's-Newman-Keuls post-test for more than two groups. A value of $p < 0.05$ was considered statistically significant.

RESULTS

We investigated the effect of increasing concentrations of carvedilol, propranolol and BM-910228 on the mitochondrial electric potential ($\Delta\Psi$) generated by succinate (Fig. 1A) and glutamate-malate (Fig. 1B). As seen, the effects of the three compounds generated the same type of curve with both substrates. We saw that carvedilol presented the greatest effect on mitochondrial $\Delta\Psi$. Propranolol had a very marginal effect and BM-910228 presented an intermediate effect. Control values for membrane potential were 229 ± 2.3 mV for glutamate-energized mitochondria and 241 ± 9.7 mV for succinate-energized mitochondria.

Higher concentrations (100 μ M) of carvedilol, propranolol and BM-910228 were used in order to study the impact on mitochondrial respiration during oxidation of external NADH by

isolated heart mitochondria. As noted above, 100 μ M of carvedilol was already described as achieving the maximal degree of inhibition⁽⁷⁾. Fig. 2 shows the results obtained by carvedilol and propranolol. BM-910228 (not shown) showed a similar effect to carvedilol. As seen in the figure, carvedilol achieved around 85% inhibition of the mitochondrial respiratory rate, while propranolol inhibited around 25% of the initial activity. NADH-sustained mitochondrial respiration was around 120-130 natms O/min/mg protein.

In order to compare the impact of carvedilol and propranolol on calcium-induced MPT, we added 500 μ M calcium to succinate-energized heart mitochondria. Fig. 3 shows a representative trace of four different mitochondrial preparations. Propranolol (10 μ M) was consistently

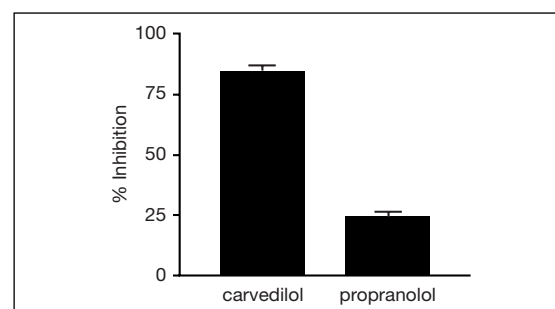


Fig. 2 Effect of propranolol and carvedilol (100 μ M) on respiratory activity sustained by 5 mM NADH. Data are presented as mean \pm SEM, from three independent experiments. Results are compared with oxygen measurements in the absence of carvedilol or propranolol. Experimental conditions are as in Fig. 1, except for the different substrates used and the absence of TPP⁺.

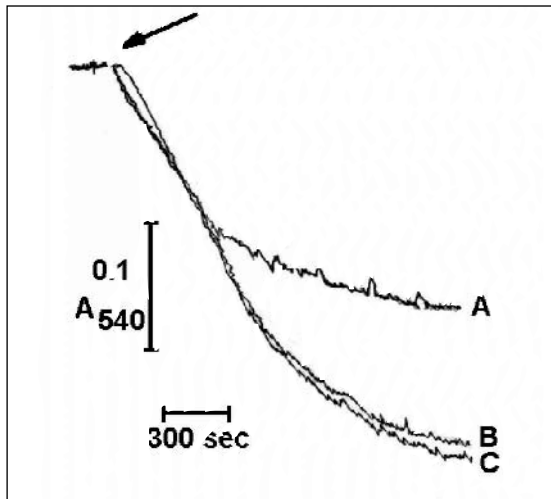


Fig 3 Typical recording of mitochondrial swelling (decrease of optical density at 540 nm) induced by calcium (500 μ M) in the presence of 10 M carvedilol (A), 10 μ M propranolol (B) and without additions (C). The mitochondria were incubated as described in the Methods section. Both drugs were pre-incubated with mitochondria for three minutes before the addition of calcium. Both the recordings are representative of four different mitochondrial preparations.

ineffective against the decrease in the suspension absorbance caused by MPT induction (the consequence of mitochondrial swelling). By contrast, carvedilol (10 μ M) showed the effect described previously⁽¹¹⁾, that is, inhibition of calcium-related MPT induction.

Since propranolol was shown to have no effect on calcium-related MPT induction, we decided to extend our previous studies on the inhibition of the MPT by carvedilol without studying the comparative effect of propranolol. Mitochondrial swelling due to MPT induction is associated with cytochrome *c* release, an event that can lead to cell apoptosis⁽¹⁷⁾. **Fig. 4** shows that calcium caused an increase in cytochrome *c* release that was partly (but significantly) inhibited by both carvedilol and cyclosporin-A (MPT inhibitor⁽²⁰⁾). As expected, incubation of heart mitochondria with a hypo-osmotic buffer caused maximal cytochrome *c* release due to rupture of the outer mitochondrial membrane. Carvedilol, without calcium, had no effect on the small release of cytochrome *c* observed in the control group (data not shown).

Another pro-oxidant substance that can be used to induce the MPT is doxorubicin, an anti-neoplastic agent that can be turned into a pro-oxidant by an NADH-related mitochondrial complex I-catalyzed reaction. In this regard, we

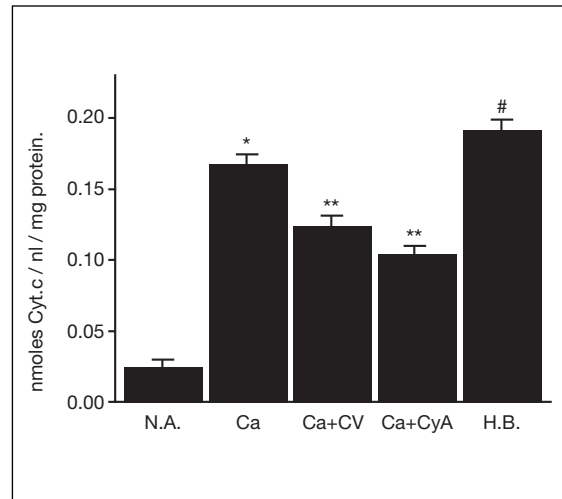


Fig. 4 Quantification of cytochrome *c* release by a spectrophotometric method. Mitochondria (4.2 mg) were incubated in 2 ml reaction buffer as described. The reactions were left running for 20 minutes before cytochrome *c* was measured. N.A.: no additions, Ca: 500 nmoles/mg protein calcium, Ca+CV: 500 nmoles/mg protein + 60 μ M carvedilol, Ca+CyA: 500 nmoles/mg protein + 3 μ M cyclosporin-A, H.B.: hypo-osmotic buffer (60 mM KCl + 10 mM HEPES). * - $p < 0.01$ vs. N.A., ** - $p < 0.05$ vs. Ca, # - $p < 0.001$ vs. N.A., $n = 3$.

used a system containing external NADH, doxorubicin, calcium and succinate to induce the MPT. As expected, carvedilol was able to inhibit the decrease in absorbance, in a dose-dependent manner (**Fig. 5**).

DISCUSSION

Carvedilol was shown previously to present protective properties in *in vitro* models of car-

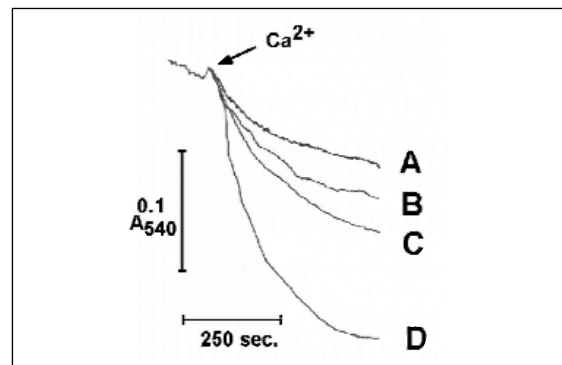


Fig. 5 Induction of the mitochondrial permeability transition by NADH, doxorubicin and calcium, as measured by decrease in mitochondrial volume. The assays were performed in 2 ml as described. Mitochondria (0.5 mg) were incubated for three minutes with doxorubicin before the addition of calcium (40 μ M). A) calcium only, B) calcium + 100 μ M doxorubicin + 20 μ M carvedilol, C) calcium + 100 μ M doxorubicin + 10 μ M carvedilol, D) calcium + 100 μ M doxorubicin. The recording is representative of three different mitochondrial preparations.

diac mitochondrial dysfunction^(5, 6, 11). Furthermore, carvedilol was shown to have a depressive effect on mitochondrial $\Delta\psi$ that was demonstrated as being caused by a weak protonophoretic effect, and neither via the activation of mitochondrial ATP-sensitive potassium channels^(13, 14) nor by significantly inhibiting the mitochondrial respiratory chain (at least for physiologically significant concentrations). In fact, the positive impact of carvedilol on cardiac mitochondrial function may be essential to increased myocyte protection during events related to excessive ROS production, like ischemia and reperfusion. It has been demonstrated that carvedilol prevents most cardiac dysfunction s u f -
ferred during and after an ischemic episode^(21, 22). Due to the importance of cardiac mitochondria in myocyte energetics, it is reasonable to state again that primary protection of mitochondrial function may be fundamental in preserving myocyte viability.

In this work, our first objective was to compare carvedilol and propranolol in three different situations involving cardiac mitochondria. The first was related to the effect of the two compounds (plus BM-910228, a metabolite of carvedilol) on mitochondrial $\Delta\psi$. The second led us to test carvedilol and propranolol in respiratory activity associated with exogenous NADH oxidation. Finally, our comparative study led us to test both carvedilol and propranolol against calcium-induced MPT, a condition known to cause mitochondrial dysfunction⁽²³⁾. The MPT has been described as being crucial in the recovery of the cardiomyocyte after an ischemic and reperfusion episode⁽²⁴⁾ and as causing outer mitochondrial rupture and leading to cytochrome *c* release⁽¹⁷⁾.

Figs. 1a and *1b* confirm the protonophoretic effect of carvedilol, as previously presented^(13, 14). In a dose-dependent manner, carvedilol was shown to decrease mitochondrial $\Delta\psi$, both in succinate- and in glutamate-malate-energized mitochondria. BM-910228, also used as comparison, showed a lesser effect, again confirming the previous idea that this metabolite has a lower protonophoretic activity⁽¹³⁾. The novelty was that propranolol (up to 40 μ M) showed no significant effect on mitochondrial $\Delta\psi$. Propranolol also showed a negligible effect in inhibiting respiratory activity related to exogenous NADH oxidation (*Fig. 2*) and in the inhibition of cal-

cium-induced mitochondrial swelling (*Fig. 3*). The last two observations are easier to discuss in terms of the advantages of carvedilol against propranolol in the protection of mitochondrial function. Note that this respiratory activity sustained by exogenous NADH was proposed to be dependent on the activity of an externally directed mitochondrial NADH dehydrogenase in rat heart mitochondria^(8, 9). It has been proposed that this enzyme produces a large amount of ROS during ischemia and reperfusion⁽²⁵⁾ and it was also demonstrated as being responsible for converting doxorubicin, an antineoplastic agent, into a highly reactive semi-quinone that would later react with oxygen to form superoxide anions⁽¹⁰⁾, with negative consequences for the myocardium. The existence of such an enzyme has been debated in the literature^(8, 9, 26). The reasons for the differing effect are not clear but they may relate to different membrane insertion. The stronger effect of carvedilol on calcium-induced MPT is something that would predictably lead to better maintenance of mitochondrial viability during myocyte oxidative stress. Although shown to have some antioxidant potential⁽¹⁶⁾, propranolol had no effect on MPT induction, indicating that direct protection of mitochondrial function via MPT inhibition is unlikely to be a mechanism for the protective effect of propranolol on the myocardium after ischemia and reperfusion⁽²⁷⁾. Regarding this point, carvedilol appears to be more advantageous. The evidence indicates that the stronger intrinsic antioxidant properties of carvedilol are a factor of difference between the two compounds.

The differences regarding the dose-dependent reduction in $\Delta\psi$ may be more open to debate. Small reductions in mitochondrial $\Delta\psi$ have been reported to reduce ROS generation by the respiratory chain⁽¹⁵⁾, something that propranolol would not be able to perform. As described, carvedilol would reduce ROS generation by the respiratory chain not only due to its antioxidant properties^(3, 4, 28) but also due to the slight reduction of mitochondrial $\Delta\psi$ (slight uncoupling) (Santos et al., manuscript in preparation). It is easily understandable that larger reductions in mitochondrial $\Delta\psi$ may compromise mitochondrial ATP synthesis. To achieve this effect, carvedilol would have to attain clinically significant concentrations. Also, at these high concentrations, carvedilol also inhibits the mitochondrial respiratory chain^(13, 14). The lower

membrane $\Delta\Psi$ could also cause a reduction in calcium entry during episodes of cytosolic calcium overload. At least *in vitro*, we observed that up to concentrations of 20 μM carvedilol does not reduce the total amount of accumulated calcium (Oliveira et al., submitted). Nevertheless, propranolol was unable to mimic the effect of carvedilol, revealing again that a direct effect on mitochondrial $\Delta\Psi$ is unlikely to be part of propranolol's protective effect after ischemia and reperfusion⁽²⁷⁾.

Due to the lack of effect of propranolol up to clinically significant concentrations, we decided to test carvedilol alone against calcium-induced cytochrome *c* release. As already described, a release of pro-apoptotic factors has been reported to occur during the MPT⁽¹⁷⁾. Among pro-apoptotic factors, cytochrome *c* is probably the best known^(29, 30). In this work, we measured the amount of cytochrome *c* released from mitochondria after a calcium insult. The amount measured was very close to that observed when a hypo-osmotic buffer was used. With this buffer, we expected to obtain rupture of the outer membrane and release of most of the membrane-bound cytochrome *c*. In the literature, it has been reported that two pools of cytochrome *c* co-exist in mitochondria, only one of them having access to the cytosol as soon as the apoptotic stimulus starts^(31, 32). In this work we observed that both carvedilol and cyclosporin-A granted partial protection against calcium-induced cytochrome *c* release (Fig. 4). Although the MPT has been correlated with the release of pro-apoptotic factors⁽¹⁷⁾, it has also been reported that in many conditions, cytochrome *c* release can occur independently of MPT induction^(33, 34). The partial protective effect of cyclosporin-A confirms that other calcium-dependent mechanisms exist for cytochrome *c* release. Concerning carvedilol, we can speculate that most of the inhibition observed was dependent on the prevention of outer mitochondrial membrane rupture but not on MPT-independent cytochrome *c* release.

It has been observed that carvedilol is able to inhibit the apoptotic process in the heart triggered by heart failure^(35, 36). Despite the existence of these studies, none of them attributed the inhibitory action of carvedilol to a direct effect on the MPT in cardiac mitochondria. We thus propose another mechanism for apoptosis inhibition by carvedilol: inhibition of the calcium-induced MPT, with consequent prevention

of outer membrane rupture.

The dose-dependent inhibitory effect of carvedilol on the MPT induced by doxorubicin and calcium confirms the mitochondrial protection afforded by carvedilol in an animal model of doxorubicin-induced cardiomyopathy⁽³⁷⁾ and the results obtained in this work and in a previous one⁽⁷⁾ regarding the inhibition of enzymatic activity proposed to be responsible for activating doxorubicin in the heart⁽¹⁰⁾. In our *in vitro* model, it is difficult to predict whether the inhibition afforded by carvedilol depends mainly on the primary inhibition of the enzyme responsible for doxorubicin activation or on its antioxidant properties instead. Nevertheless, the results again show that carvedilol shows advantages in counteracting doxorubicin's toxic effects on the myocardium.

In conclusion, carvedilol appears to have a more direct protective effect against cardiac mitochondrial dysfunction, thus demonstrating that in the context of protection of mitochondrial function, beta-adrenergic antagonism is not an important factor. In a clinical context, it appears that carvedilol may have this extra advantage in maintaining myocyte viability. The protection of mitochondrial function after cardiac ischemia and reperfusion observed with propranolol⁽²⁷⁾ may be due to hemodynamic-based protection and not to direct effects on mitochondrial function. With the results shown, we also show more arguments that demonstrate that carvedilol is effective in several models of mitochondrial dysfunction, particularly those in which mitochondrial damage is oxidative damage-dependent.

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