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Carvedilol improves energy production during acute global myocardial ischaemia☆

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Abstract

Cardiac mitochondria may become dysfunctional during ischaemia, thus compromising cardiomyocyte function. Carvedilol is an α_1/β-adrenoceptor antagonist with antioxidant, neuroprotective, cardioprotective and vascularprotective properties, and is used to treat hypertension, myocardial ischaemia and congestive heart failure. However, its impact on mitochondrial function during acute prolonged ischaemia is unknown. We aimed to study the effect of carvedilol on cardiac mitochondrial function during acute ischaemia, using Wistar rat hearts perfused with a Langendorff system, and then submitted to ischaemia in the presence and absence of carvedilol. We determined the electrical potential of the mitochondrial membrane, O_2 consumption by the respiratory chain, energy charge and the activity of the mitochondrial respiratory chain complexes. In our model, carvedilol had a preferential action on phosphorylation, increasing the mitochondrial energy charge (0.76 ± 0.03 vs. 0.65 ± 0.01 arbitrary units; $P < 0.05$) and decreasing the phosphorylation lag phase (28.64 ± 4.23 vs. 62.4 ± 11.63 s; $P < 0.05$) during ischaemia. The larger amount of energy available allowed the preservation of the electrical potential (201.2 ± 2.45 vs. 186.66 ± 3.36 mV;$P < 0.05$), thus improving mitochondrial function during acute prolonged ischaemia.

Keywords: Ischaemia; Carvedilol; Mitochondrion

1. Introduction

In Western countries, ischaemic heart disease is one of the leading causes of morbidity and mortality. Although this pathology has been recognised for centuries, only recently have we started to understand its cellular and molecular basis. During cardiac ischaemia, there is a relative or absolute shortage of oxygen and nutrients in cardiomyocytes, resulting in an impairment of energy production by the mitochondria. Without energy, the proper structure and function of cardiomyocytes and their components are at risk.

Energy shortage and, therefore, cardiac mitochondria are at the centre of the metabolic dysfunctions induced by ischaemia. Thus, it is important to develop and use drugs able to preserve the mitochondrial energy production during ischaemia.

Carvedilol is a β- and α_1-adrenoceptor antagonist. These unique combined properties make it a vasodilator (increasing coronary blood flow) and a negative inotrope (reducing cardiac contractility)—both these actions are useful to reduce the negative impact of ischaemia on cardiac metabolism. Carvedilol is currently used to treat hypertension (Dunn et al., 1997; Feuerstein and Ruffolo, 1995; Noguchi et al., 2000), myocardial ischaemia (Ryan et al., 1999) and congestive heart failure (Cleland and McGowan, 1999; Cleland et al., 1999; Colucci et al., 1996; Dargie, 2000, 2001; Dargie et al., 1999; Feuerstein et al., 1998; Packer et al., 1996, 2001). Several authors have already shown that this drug has neuroprotective (Lysko et al., 1992a,b; Yue et al., 1994a), cardioprotective (Feuerstein et al., 1993; Yaoita et al., 2002) and vascularprotective (Yue et al., 1993, 1994b)

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properties. It is also known that carvedilol can act as an antioxidant (Yue et al., 1992). In animal models, carvedilol was able to markedly decrease the area of infarct, mainly in those models where it is known that the production of reactive oxygen species is important to induce and expand an area of acute ischaemia (Ruffolo et al., 1993).

These positive findings also occurred in human studies. CAPRICORN (Carvedilol Post infarct survival Control in left ventricular dysfunction) was a large clinical trial (1959 patients) that studied the impact of carvedilol (6.25 mg bid, uptitrated to 25 mg bid) on the mortality and morbidity of patients with left ventricular dysfunction after an acute myocardial infarction; treatment was initiated before hospital discharge and less than 21 days after acute myocardial infarction (Cleland et al., 1999; Dargie, 2000; Dargie et al., 1999). In this study, patients treated with carvedilol showed a significant decrease in total mortality, cardiovascular mortality and nonfatal reinfarction (Dargie, 2001). However, the ultrastructural basis of the positive effect of carvedilol on ischaemic hearts is still to be completely determined. We hypothesised that at least part of the results shown by carvedilol in clinical trials involving patients with coronary artery disease are due to a positive impact on mitochondrial energy production during episodes of ischaemia.

Our work had, therefore, the objective of studying the influence of carvedilol on the mitochondrial function of rat hearts exposed to acute ischaemia, using an ex vivo model of perfusion with a Langendorff system. Our hypothesis was that carvedilol may prevent mitochondrial damage, thus allowing the myocyte to be supplied with mitochondrial ATP after the ischaemic period.

2. Material and methods

This investigation conformed with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health-NIH publication No. 85-23, revised 1996-(National Research Council, 1996) and it was approved by the Ethics Committee of the Centre for Neuroscience and Cell Biology of Coimbra, where the experimental work took place. All compounds were of the purest quality available and were ordered from Sigma (St. Louis, MO) or Merck (Darmstadt, Germany), except for carvedilol, which was obtained from Roche Diagnostics (Mannheim).

2.1. Experimental groups

Thirty Wistar rats were randomly assigned into three experimental groups: control ($n=10$), ischaemia ($n=10$) and ischaemia + carvedilol 40 μM ($n=10$).

2.2. Assembly of the ex vivo perfusion system

Wistar rats, weighing around 300 g, were killed by CO_2 inhalation. Each heart was rapidly excised and mounted in a Langendorff perfusion apparatus (model UP-100 from Hugo Sachs Elektronik, Germany), and then perfused at a constant flow (25 ml/min) with a Krebs modified solution (NaCl 118 mM, KCl 4.7 mM, KH_2PO_4 1.2 mM, $NaHCO_3$ 25 mM, $MgSO_4$ 1.2 mM, Na_2EDTA 0.5 mM, Glucose 10 mM and $CaCl_2$ 3 mM; pH 7.4), gassed with carbogen (95% O_2–5% CO_2). Temperature was continuously monitored and maintained at 37 °C (via a thermostatic bath) throughout the perfusion period (180 min for the control group and 60 min for the remaining groups); pH was also continuously monitored with a pH electrode and kept stable at 7.35–7.45 during this period. Carvedilol (40 μM) was added to the Krebs modified solution in the respective experimental group. Left ventricular pressure and heart rate were recorded at regular intervals.

After the initial perfusion, all the rat hearts, with the exception of the control group, were submitted to 120 min of ischaemia at 37 °C in a solution identical to that of the perfusion period, but without glucose and carbogen (replaced by nitrogen).

2.3. Preparation of the biological material

2.3.1. Isolation of rat heart mitochondria

At the end of the perfusion and ischaemia periods, the mitochondrial fraction from each rat heart was prepared according to the method described by Rickwood et al. (1987a). All the parameters of mitochondrial function were assessed in the isolated mitochondrial fraction.

2.4. Laboratory methods used

2.4.1. Determination of protein concentration

The protein concentration of the mitochondrial fraction was determined using the biuret colorimetric method (Gornall et al., 1949).

2.4.2. Determination of the mitochondrial membrane potential

Membrane electrical potential ($\Delta\Psi$) was determined through the permanent recording of the distribution of the lipophilic cation tetraphenylphosphonium (TPP^+) across the inner mitochondrial membrane. This was done with an electrode sensitive to TPP^+ prepared in our laboratory, according to the principles established by Kamo et al. (1979). Reactions took place at a constant temperature of 25 °C, in an open and thermostable reaction chamber, using 1 ml of reaction solution (sucrose 130 mM, KCl 50 mM, $MgCl_2$ 5 mM, KH_2PO_4 5 mM and (*N*-[2-hydroxyethyl] piperazine-*N*′ -[2-ethanosulphonic]) acid (HEPES) 5 mM, pH 7.2), supplemented with 3 μM TPP^+ and the mitochondrial suspension (volume corresponding to 2 mg of protein, as determined by the biuret method). The determination of mitochondrial $\Delta\Psi$ was performed using the method described by Muratsugu et al. (1977) and Kamo et al. (1979). The matrix volume was assumed to be 1.1 μl/mg of protein.

2.4.3. Evaluation of mitochondrial respiratory activity

All the assays were performed at 25 °C, in a closed reaction chamber, with stable temperature and 1 ml capacity.

Oxygen consumption was evaluated polarographically with an O_2 electrode (Yellow Springs Instruments), Clark type, connected to a Kipp and Zonen recorder, through a command unit manufactured at our laboratory. Electrode calibration was done according to the protocol described by Rickwood et al. (1987b).

Reactions were initiated by adding the mitochondrial suspension to the reaction solution (as previously described). The later addition of the respiratory substrate [glutamate 10 mM + malate 5 mM, succinate 5 mM or ascorbate 5 mM + *N*,*N*,*N*′,*N*′ -tetramethyl-P-phenylenodiamine (TMPD) 0.25 mM] induced an increase in O_2 consumption by the mitochondrial fraction. Respiratory state 3 (characterised by a higher and faster O_2 consumption) was then induced by the addition of adenosine diphosphate (ADP) 250 nmol (62.5 nmol when using ascorbate/TMPD). After all ADP was consumed, the reaction returned to a slower velocity, respiratory state 4. Respiratory Control Ratio was evaluated as the quotient between state 3 and state 4.

2.4.4. Evaluation of the energy charge

The protocol for extraction of the adenine nucleotides (ATP, ADP and AMP) was as follows: at the end of one phosphorylation cycle induced by the addition of ADP, 250 μl of the final respiratory medium was collected (corresponding to 0.5 mg of mitochondrial protein) and added to 250 μl of $HClO_4$ 0.6 M (with 25 mM of EGTA). Five minutes later, the mixture was agitated in a vortex (to obtain a protein-free sample) and centrifuged for 2 min at 10,000 × *g* in an Eppendorf centrifuge (at 0 °C). The pellet was then removed and the supernatant was neutralised with KOH 3 M + Tris 1.5 M and then centrifuged at 10,000 × *g* for another 2 min. All procedures were conducted at low temperature (0–4 °C). The adenine nucleotides were separated by high-pressure liquid chromatography (HPLC), reverse phase (Stocchi et al., 1985), in a Beckman Gold chromatograph, with a model 126 pump and a variable UV detector (model 166), controlled by computer. The detection wavelength was 254 nm, using a Licrosphere 100 RP-18 column (5 mm) made by Merck. The protocol used consisted in isocratic elution with potassium phosphate buffer (100 mM; pH 6.5) and methanol 1%. The flow rate was 1.25 ml/min for 5 min (to achieve nucleotide separation); the detection limit for each compound was 3–5 pmol.

The energy charge was determined according to the following formula: ([ATP] + 0.5*[ADP]/([ATP]+[ADP]+[AMP])).

2.4.5. Determination of enzyme activity

2.4.5.1. Complex I. The activity of this enzyme complex was evaluated by spectrofluorometry: complex I converts NADH (reduced nicotinamide dinucleotide) into NAD^+ (oxidised nicotinamide dinucleotide), and pyridine nucleotides produce fluorescence at 450 nm, when excited at 366 nm. Disrupted mitochondria (disruption induced by freezing/thawing cycles) do not possess an internal pool of reduced nucleotides (like NADH). Therefore, the heart mitochondria obtained by differential centrifugation were submitted to three cycles of freezing/thawing. Afterwards, a volume of the mitochondrial fraction corresponding to 0.4 mg of protein (as determined by the biuret method) was placed in quartz cuvettes containing 2 ml of the buffer solution (KH_2PO_4 25 mM + $MgCl_2$ 10 mM; pH 7.4) and KCN 1 mM. Fluorescence emission was determined in a Perkin Elmer spectrofluorimeter (LS 50B model) at 30 °C; the reaction was initiated by the addition of NADH 50 μM. After the emission peak was measured, dodecylubiquinone 162.5 μM was added, with a consequent fall in fluorescence emission, graphically expressed by a line whose slope allowed the determination of mitochondrial complex I activity. In the final phase of each assay, a specific complex I inhibitor (rotenone 3.8 μM) was added, in order to guarantee that the previously obtained values were indeed complex I enzyme activity. Enzyme activity was the difference between the slopes of the lines before and after rotenone addition, determined with the FL Winlab software provided with the spectrofluorimeter. This value is expressed in arbitrary units.

2.4.5.2. Complexes II–III. In order to evaluate the activity of complexes II–III, the method described by King (1967) was used. Enzyme activity (in nmol/min/mg of protein) was determined with the following equation: AE=[(ΔAbs)/19.2]*(1000/μl of mitochondrial suspension)*1000 in which AE represents the enzyme activity and ΔAbs represents the absorbance variation per minute at 550 nm. Results are expressed as nmol of succinate oxidized/min/mg protein.

2.4.5.3. Complex IV. For the determination of complex IV enzyme activity, O_2 consumption associated with cytochrome *c* oxidation by complex IV was measured. Rotenone (specific inhibitor of complex I) and antimycin A (inhibitor of complexes II–III) were added to the reaction medium, in order to guarantee that the O_2 consumption was not due to the activity of other mitochondrial enzyme complexes. This measurement was made with the technique already used for the determination of mitochondrial respiratory activity. The same reaction medium was used, to which rotenone 3 μM, antimycin A 0.5 μg, a volume of mitochondrial suspension corresponding to 0.1 mg of protein and cytochrome *c* 15 μM were sequentially added.

The reaction was initiated with 10 μl of Ascorbate 500 mM/TMPD 25 mM, and O_2 consumption was registered in the same way as for the determination of respiratory activity. O_2 consumption was used to determine complex IV enzyme activity, expressed in nmol O_2/min/mg protein.

2.4.5.4. ATP synthase. To determine the activity of this enzyme complex, assays were conducted at 37 °C in an open reaction chamber under permanent shaking, in a volume of 2 ml of the reaction medium (sucrose 130 mM, KCl 60 mM, HEPES 0.5 mM and $MgCl_2$ 2.5 mM; pH 7.0). After 1 min, the time necessary to achieve the desired buffer temperature, rotenone 3 μM and 0.5 mg mitochondrial protein were added. The reaction was initiated with ATP-Mg^{2+} 2 mM, and pH variations were evaluated continuously by a protonometric method (Madeira et al., 1974), using a Crison pH evaluation system consisting of a glass electrode connected to a Kipp and Zonen recorder. At the end of the reaction, pH titration was performed, using 10 mM HCl and KOH. Enzyme activity was calculated using the slope of the curve for change in pH after the addition of ATPMg^{2+} and is expressed in nmol H^+/min/mg protein. Oligomycin was not used because our preparations were essentially devoid of myofibrils or other cellular debris, as determined by electron microscopy (data not shown).

2.5. Statistical analysis

Results are presented as means ± standard error (raw data or expressed as percentage of control), for the number of results indicated. Results were analyzed using the one-way ANOVA test. The level of significance used was $P < 0.05$.

3. Results

Our results showed that oxidative phosphorylation was very sensitive to ischaemia, as demonstrated by the decrease in the respiratory control ratio, when compared with the value obtained for control group mitochondria (2.68 ± 0.25 vs. 1.51 ± 0.13; $P < 0.05$). In the group treated with carvedilol, there was no significant improvement in respiratory control ratio (1.61 ± 0.08 vs. 1.51 ± 0.13—that is, 60.1 ± 3% vs. 56.3 ± 4.9% in the group submitted to ischaemia without carvedilol; P = n.s.; Fig. 1).

When glutamate/malate was used as energy substrate, electrical potential ($\Delta\Psi$) values decreased significantly in the ischaemic group, when compared to the control group (209.13 ± 1.37 mV vs. 186.66 ± 3.36 mV; $P < 0.001$); a similar result was found with succinate (211.32 ± 3.24 mV vs. 187.23 ± 4.74 mV; $P < 0,05$) and ascorbate/TMPD (209.09 ± 2.53 mV vs. 189.24 ± 3.98 mV; $P < 0.05$). Mitochondria from hearts treated with carvedilol 40 μM developed a significantly higher electrical potential than did mitochondria from hearts exposed to ischaemia in the absence of carvedilol. This was observed for all three energy substrates used: glutamate/malate (95.4 ± 1.2% in the carvedilol group vs. 89.3 ± 1.6% in the ischaemic group—201.2 ± 2.45 mV vs. 186.66 ± 3.36 mV; $P < 0.05$), succinate (94.9 ± 1.2% in the carvedilol group vs. 88.6 ± 2.2% in the ischaemic group or 200.73 ± 2.6 mV vs. 187.23 ± 4.74 mV; $P < 0.05$) and ascorbate/TMPD (96.5 ± 1.4% in the carvedilol group vs. 90.5 ± 1.9 in the ischaemic group—201.87 ± 2.85 mV vs. 189.24 ± 3.98 mV; $P < 0.05$; Fig. 2A–C).

Another parameter evaluated was the amount of time needed to phosphorylate a fixed amount of ADP (250 nmol)-lag phase. The lower this value is, the quicker the phosphorylation cycle is completed. We observed a longer lag phase in the ischaemic group, when compared to control, not only with glutamate/malate (59.08 ± 6.82 vs. 127.2 ± 19.03 s; $P < 0.05$), but also with succinate (66.46 ± 6.43 vs. 275.73 ± 45.99 s; $P < 0.05$) and ascorbate/TMPD (31.5 ±

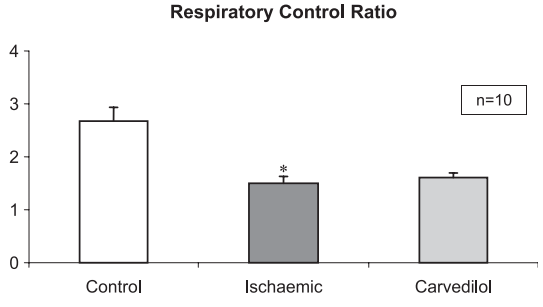

Fig. 1. Comparison of respiratory control ratio values in rat heart mitochondria (control, ischaemic and carvedilol groups) with glutamate/malate as energy substrate. Values are presented as a ratio.

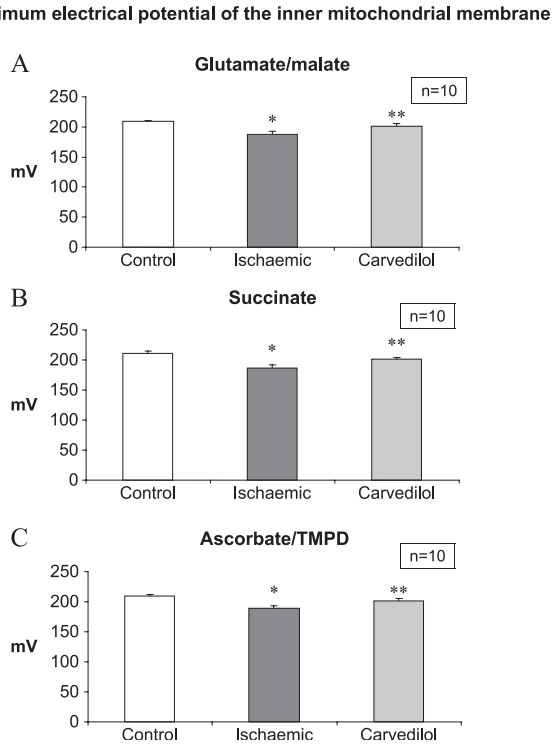

Fig. 2. Comparison between maximal electrical potential ($\Delta\Psi$) in the control, ischaemic and carvedilol-treated hearts, after the addition of the energy substrate to the reaction medium containing rat heart mitochondria: (A) glutamate/malate; (B) succinate; (C) ascorbate/TMPD. *$P < 0.05$ vs. the ischaemic group.

2.11 vs. 62.4 ± 11.63 s; $P < 0.05$). The group treated with carvedilol showed, globally, a shortening of the lag phase time (vs. ischaemic group), but of different magnitude, depending on the substrate used. When glutamate/malate was used, the lag phase decrease was minimal (204.5 ± 12.7%–120.82 ± 7.53 s–in the carvedilol group vs. 215.3 ±32.2%–127.2 ± 19.03 s–in the ischaemic group; P = n.s.). It was greater, although still non-significant, with succinate (337.2 ± 24.6%–224.54 ± 15.99 s–in the carvedilol group vs. 414.9 ± 69.2%–275.73 ± 45.99 s–in the ischaemic group; P = n.s.) and only became significant when complex IV was specifically activated with ascorbate/TMPD (90.9 ± 13.4%–28.64 ± 4.23 s–in the carvedilol group vs. 198.1 ± 6.7%–62.4 ± 11.63 s–in the ischaemic group; $P < 0.05$; Fig. 3A–C).

As already mentioned, the energy charge ([ATP]+0.5∗[ADP]/([ATP]+[ADP]+[AMP])) was calculated for each substrate used. The ischaemic group had a lower energy charge, when compared to the control group, regardless of the energy substrate evaluated: glutamate/malate (0.965 ± 0.005 vs. 0.89 ± 0.04; P = n.s.), succinate (0.79 ± 0.051 vs. 0.65 ± 0.01; $P < 0.05$) or ascorbate/TMPD (0.7 ± 0.04 vs. 0.59 ± 0.02; P = n.s.); however, this difference only reached statistical significance when succinate was used as substrate. The energy charge of mitochondria from hearts treated with carvedilol was not significantly different from that of mitochondria from the ischaemic group when glutamate/malate (86 ± 4.1%–0.83 ± 0.04–in the carvedilol group vs. 92.2 ± 4.1%–0.89 ± 0.04–in the ischaemic group; P = n.s.) was used as substrate. However, when using succinate, the group treated with carvedilol was able to generate a significantly higher energy charge (96.2 ±3.8%–0.76 ± 0.03–in the carvedilol group vs. 82.3 ±1.3%–0.65 ± 0.01–in the

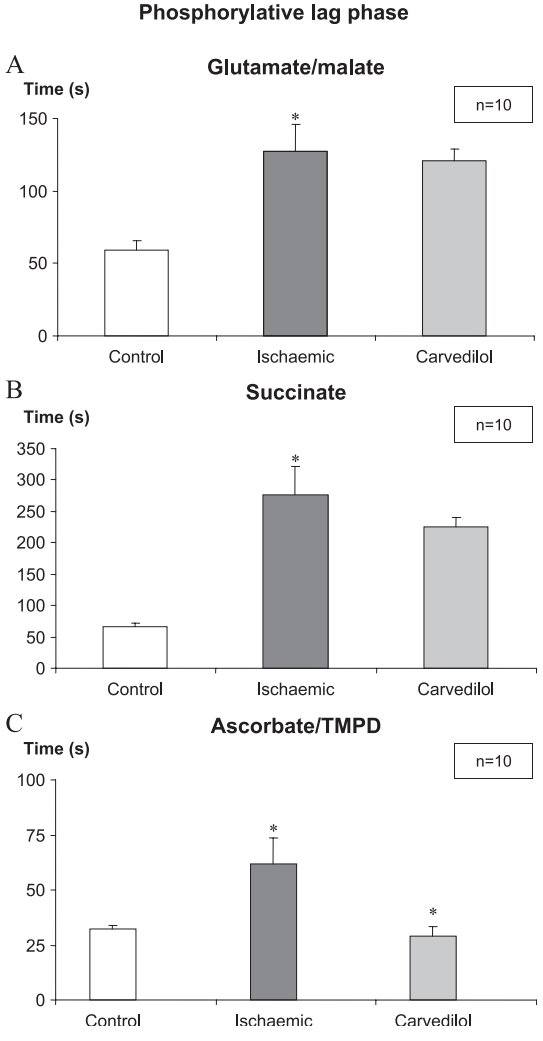

Fig. 3. Comparison of the phosphorilation lag phase in the three experimental groups (control, ischaemic and carvedilol) for each of the energy substrates studied, after the addition of 250 nmol of ADP (62.5 when using ascorbate/TMPD) to the medium containing rat heart mitochondria and the substrate: (A) glutamate/malate; (B) succinate; (C) ascorbate/TMPD. *$P < 0.05$ vs. the ischaemic group.

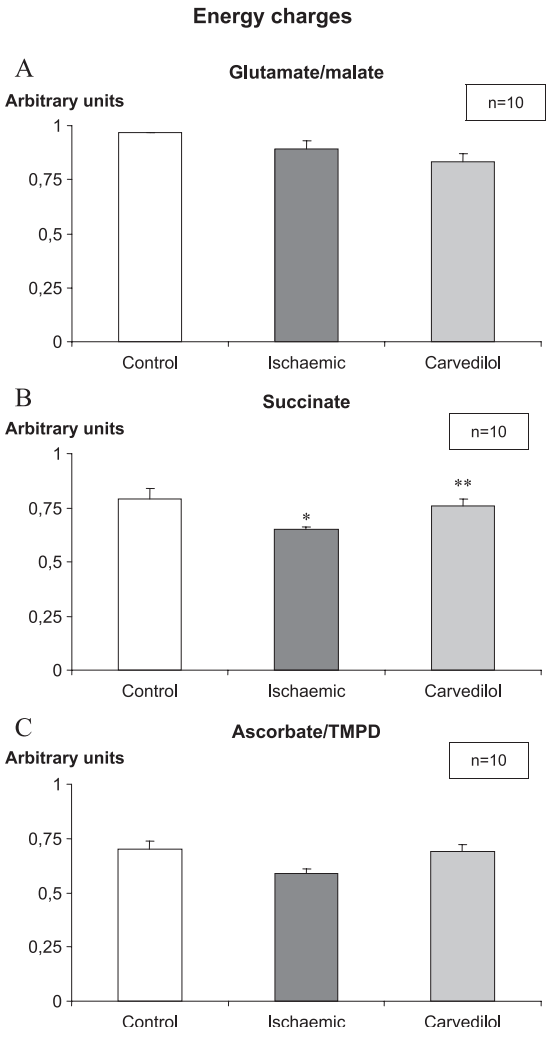

Fig. 4. Comparison of the energy charge values (obtained by HPLC) in the three experimental groups (control, ischaemic and carvedilol) for each of the energy substrates studied, after the addition of 250 nmol of ADP (62.5 when using ascorbate/TMPD) to the medium containing rat heart mitochondria and the energy substrate: (A) glutamate/malate; (B) succinate; (C) ascorbate/TMPD. *$P < 0.05$ vs. the ischaemic group.

ischaemic group; $P<0.05$). The same was found when ascorbate/TMPD was used (98.6 ±4.3%–0.69 ± 0.03–in the carvedilol group vs. 84.3 ± 2.9%–0.59 ± 0.02–in the ischaemic group; $P<0.05$; Fig. 4A–C).

The activity of the respiratory chain complexes I, II–III, IV and ATP synthase was also evaluated. A statistically significant difference ($P<0.05$) was found between ischaemic and control groups for all four enzyme complexes evaluated (complex I: 100 vs. 63.23 ± 5.18 arbitrary units; complexes II–III: 14,370 ± 1174 vs. 8592 ± 663 nmol succinate oxidized/min/mg protein; complex IV: 853 ± 66 vs. 608 ± 58 nmol O_2 consumed/min/mg protein; ATP synthase: 795 ± 75 vs. 426 ± 35 nmol H^+ released/min/mg protein). The carvedilol group showed a trend towards a higher activity for all complexes, although without reaching statistical significance. The mean values obtained were as follows: complex I 66.2 ± 8.8 arbitrary units in the carvedilol group vs. 63.23 ± 5.18 arbitrary units in the ischaemic group (P=n.s.); complexes II–III 66 ± 13% (9485 ± 1863 nmol succinate oxidized/min/mg protein) in the carvedilol group vs. 59.8 ± 4.6% (8592 ± 663 nmol succinate oxidized/min/mg protein) in the ischaemic group (P=n.s.); complex IV 76.1 ± 6.1% (649 ± 52 nmol O_2 consumed/min/mg protein) in the carvedilol group vs. 71.3 ± 6.8% (608 ± 58 nmol O_2 consumed/min/mg protein) in the ischaemic group (P=n.s.); and ATP synthase 63.1 ± 7.3% (502 ± 58 nmol H^+ released/min/mg protein) in the carvedilol group vs. 53.6 ± 4.4% (426 ± 35 nmol H^+ released/min/mg protein) in the ischaemic group (P=n.s.; Fig. 5A–D).

4. Discussion

This work was performed to study the impact of carvedilol on the mitochondrial function of rat hearts exposed to acute ischaemia. Our working hypothesis was that carvedilol could prevent mitochondrial damage, possibly by means of its intrinsic antioxidant properties. The chosen carvedilol concentration was within the range of doses shown in the literature to have maximal antioxidant effects without being toxic to mitochondria (Abreu et al., 2000). The results showed that this drug had a different impact on the mitochondrial function parameters assessed.

Regarding the activity of the mitochondrial respiratory chain and coupling of oxidative phosphorylation, the direct effect of carvedilol was not significant. In fact, respiratory control ratio values obtained in the carvedilol group were similar to those obtained for the ischaemic group. The same was true for the enzyme activity of the respiratory chain components: values obtained for the carvedilol group were not significantly different from those for the ischaemic group. When analysing these results, it is important to remember that, when using an experimental model of acute

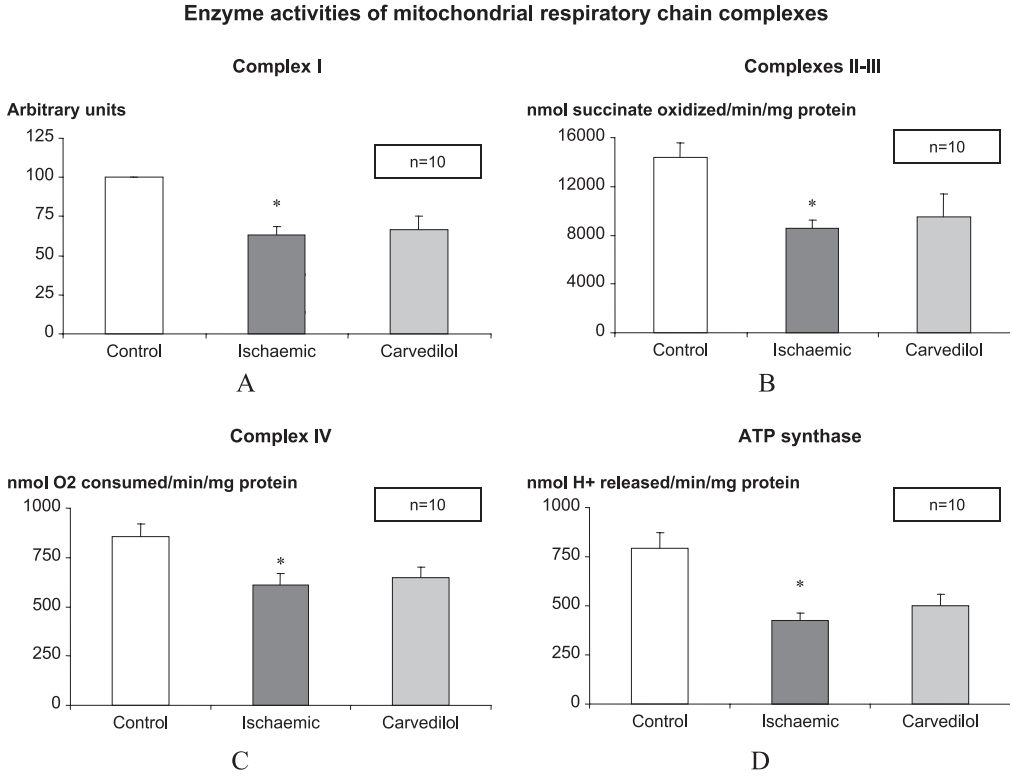

Fig. 5. Comparison of the values of the enzyme activity of rat heart mitochondria of the three experimental groups (control, ischaemic and carvedilol), for each of the complexes studied (I, II–III, IV e ATP synthase), in the presence of the substrate of each complex (NADH, succinate, ascorbate/TMPD or ATP-Mg^{2+}, respectively): (A) complex I; (B) complexes II–III; (C) complex IV; (D) ATP synthase. *$P<0.05$ vs. the ischaemic group.

prolonged perfusion (the control hearts were perfused during 180 min), a significant deterioration of mitochondrial function is to be expected when compared with that of control hearts whose mitochondria are isolated immediately after the animal is killed (Crestanello et al., 2002a,b). Therefore, a direct comparison between the respiratory control ratio values obtained in control mitochondria and in mitochondria from hearts not submitted to perfusion (or only submitted to short-term perfusion) is not possible, as the conditions are not equivalent.

The most favourable effects of carvedilol were on the functional status of the phosphorylation system. Carvedilol was able to decrease the length of the lag phase and to increase the mitochondrial energy charge. This positive impact on cardiac mitochondrial phosphorylation during ischaemia (making it faster—shorter lag phase—and leading to higher ATP production—higher energy charge), allowed it to generate greater energy reserves, which are important to obtain a better control of the mitochondrial transmembrane electrochemical gradient, resulting in a higher electrical potential ($\Delta\Psi$) in the carvedilol group, regardless of the substrate used.

In experimental models involving non-ischaemic hearts, carvedilol induced a slight decrease in mitochondial electrical potential, which the authors attributed to a protonophoretic effect (Abreu et al., 2000; Oliveira et al., 2000); however, it should be noted that it has not been demonstrated that such an effect also occurs during ischaemia. An alternative hypothesis is that, during ischaemia, this protonophoretic effect can be supplanted by the already demonstrated antioxidant effect of carvedilol, allowing the protection of the mitochondrial membranes from oxidation and lipoperoxidation (Oliveira et al., 2001; Rolo et al., 2001; Santos and Moreno, 2001).

However, results obtained in our experimental model can only be partially explained by the antioxidant effect of carvedilol. It is known that the production of reactive oxygen species during isolated ischaemia (not followed by reperfusion) is modest (Jassem et al., 2002), and that when oxygen is reintroduced in the system (during reperfusion), the production of reactive oxygen species increases sharply. So, in a model of ischaemia without reperfusion, a positive impact on cardiac mitochondrial function can not be attributed solely to an antioxidant action. Recent experimental work performed with animal models of coronary stenosis and coronary occlusion suggested that, in acute ischaemia settings without reperfusion, carvedilol was unable to prevent ventricular remodelling (a feature usually related to its antioxidant properties) (Yaoita et al., 2002).

When considered globally, the results obtained for the carvedilol group in this experimental protocol allow us to suggest that, during acute prolonged ischaemia (as in the ischaemic and carvedilol groups), the protective effect of carvedilol on the mitochondrial respiratory chain (via its antioxidant activity) may not be pronounced. Instead, carvedilol had a protective effect on the phosphorylation system, thus allowing a greater and faster generation of ATP as soon as oxygen became available (as in our in vitro system for measuring mitochondrial function), which is essential to the preservation of cardiac mitochondrial function (and, lato senso, to the survival of cardiomyocytes) in the context of an ischaemic insult.

Our data are concordant with the results of large clinical trials that assessed the impact of antioxidant drugs in the treatment of myocardial ischaemia. To date these clinical trials have been unable to prove a positive effect on mortality in patients with coronary artery disease (GISSI-Prevenzione Investigators (Gruppo Italiano per lo Studio della Sopravvivenza nell'Infarto Miocárdico), 1999; Heart Protection Study Collaborative Group, 2002; The Heart Outcomes Prevention Evaluation Study Investigators, 2000; Stephens et al., 1996). What really distinguishes carvedilol from these drugs is its ability to generate more ATP without increasing O_2 consumption. We propose that carvedilol may act during ischemia to protect the phosphorylation system, so that the system can produce large amounts of ATP quickly, as soon as the respiratory chain is again exposed to oxygen, thus solving the main problem accompanying ischaemia—the shortage of energy for myocyte recovery after reperfusion.

Our work clearly shows, for the first time, that during episodes of acute myocardial ischaemia the cardioprotective effect of carvedilol may be due to a positive impact on the mitochondrial phosphorylation system, allowing a faster and greater production of energy during the later reperfusion phase. The extra energy supply is essential to the preservation of cellular viability during the reperfusion phase that follows the ischaemic insult. This represents a clear difference from ischaemia/reperfusion settings, where carvedilol seems to act mainly as an antioxidant (Oliveira et al., 2002). This knowledge allows a better understanding of the complex mechanisms behind the positive impact of carvedilol in patients with coronary artery disease.

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