Reduction of oxidative stress by carvedilol: role in maintenance of ischaemic myocardium viability

Anna Cargnoni, Claudio Ceconi, Palmira Bernocchi, Antonella Boraso, Giovanni Parrinello, Salvatore Curello, Roberto Ferrari

Cardiovascular Research Centre, Fondazione Salvatore Maugeri, IRCCS, Via Pinidolo 23, Gussago, Brescia, Italy
Chair of Cardiology, University of Brescia, Brescia, Italy
Statistical Department, University of Brescia, Brescia, Italy
Chair of Cardiology, University of Ferrara, Ferrara, Italy

Received 2 December 1999; accepted 23 March 2000

Abstract

Objectives: To differentiate the impact of the β-blocking and the anti-oxidant activity of carvedilol in maintaining myocardium viability. Methods: Isolated rabbit hearts, subjected to aerobic perfusion, or low-flow ischaemia followed by reperfusion, were treated with two doses of carvedilol, one dose (2.0 μM) with marked negative inotropic effect due to β-blockage and the other (0.1 μM) with no β-blockage nor negative inotropism. Carvedilol was compared with two doses of propranolol, 1.0 without and 5.0 μM with negative inotropic effect. Anti-oxidant activity was measured as the capacity to counteract the occurrence of oxidative stress and myocardium viability as recovery of left ventricular function on reperfusion, membrane damage and energetic status. Results: Carvedilol counteracted the ischemia and reperfusion induced oxidative stress: myocardial content of reduced glutathione, protein and non-protein sulfhydryl groups after ischaemia and particularly after reperfusion, was higher in hearts treated with carvedilol, while the myocardial content of oxidised glutathione was significantly reduced (0.30 ± 0.03 and 0.21 ± 0.02 vs. 0.39 ± 0.03 nmol/mg prot, both P < 0.01, in 0.1 and 2.0 μM). At the same time, carvedilol improved myocardium viability independently from its β-blocking effect. On the contrary, propranolol maintained viability only at the higher dose, although to a lesser extent than carvedilol. This suggests that the effects of propranolol are dependent on energy saving due to negative inotropism. The extra-protection observed with carvedilol at both doses is likely due to its anti-oxidant effect. Conclusions: Our data show that the anti-oxidant activity of carvedilol is relevant for the maintenance of myocardium viability. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: Adrenergic (ant)agonists; Free radicals; Ischemia; Ventricular function

1. Introduction

Besides their use in hypertension and myocardial infarction (MI), β-blockers have been considered in the therapy of post-ischaemic left ventricular (LV) dysfunction and heart failure (HF) [1,2]. Their efficacy in these conditions cannot be explained only by the β-blocking activity: other 'ancillary' properties, such as the anti-oxidant activity, may be important [3].

Carvedilol is an effective β-blocking cardioprotective agent with α-blocking and anti-oxidant properties [3–5]. The anti-oxidant action may be relevant to preserve myocardium viability, particularly during ischaemia and reperfusion, conditions linked to the occurrence of oxidative stress [6–13].

Thus, the aim of our study was to differentiate the impact of the β-blocking and the anti-oxidant activities in the carvedilol-induced maintenance of viability of the ischaemic myocardium. Previous studies were performed using carvedilol’s doses which, by exerting negative...
inotropic effects, reduced heart work [7,8,11,12]. Thus, these studies could not discriminate whether the preserved anti-oxidant state by carvedilol was secondary to its cardio-protective activity — due to energy saving — or played an active role by itself.

We used isolated crystalloid perfused paced rabbit hearts, as this model clearly show the β-blocking activity in terms of negative inotropic effect. Moreover, by using different doses of the tested drug, with or without negative inotropism, we tried to determine whether the anti-oxidant properties of carvedilol contributed to its cardio-protective effect.

In accordance with the experimental design, we determined:

1. Myocardium viability, measured in terms of: (a) LV function assessed as systolic, diastolic and coronary perfusion pressures, (b) membrane damage assessed as creatine phosphokinase (CPK) release, and (c) cellular energetic status assessed as high energy phosphate content and cellular redox status
2. Oxidative stress, inferred as the ‘anti-oxidant/oxidant status’ which was measured by reduced and oxidised glutathione (GSH and GSSG, respectively) metabolism and sulfhydryl group (SH) contents.

In addition, we compared the effects of carvedilol with those of propranolol, used at two dosages: these dosages did not possess any anti-oxidant effect [14,15], and one of them exerted a negative inotropic effect.

2. Methods

The study was performed in accordance with European Union guidelines (86/609/CEE) for the use and care of laboratory animals.

2.1. Perfusion of the hearts

A total of 126 male New Zealand white rabbits (2.0–2.3 kg), maintained on a standard diet, were used. They were stunned by a blow to the neck. The hearts were quickly removed and placed in ice-cold perfusion buffer (4°C) and perfused as previously described [16]. The perfusion solution was warmed to 37°C, bubbled with 95% O₂ and 5% CO₂, and delivered to the heart with a Gilson Minipuls 2 rotary pump providing a coronary flow (CF) of 25 ml/min. Hearts were jacketed (40–44°C) to provide a constant myocardial temperature of 37°C independently from CF. The hearts were paced at a rate of 180 bpm [16]. LV function and coronary perfusion pressure were monitored as described [16].

2.2. Experimental conditions

After 45 min of aerobic equilibration (time zero as baseline condition) with CF of 25 ml/min, the hearts were subjected to: (a) 90 min of aerobic perfusion, or (b) 60 min of global, severe low-flow ischaemia (CF=0.5 ml/min), or (c) 60 min of ischaemia followed by 3 min of reperfusion at CF of 25 ml/min, or (d) 60 min of ischaemia followed by 30 min of reperfusion. LV function was monitored and 2.5-ml aliquots of coronary effluent were timely collected. At the end of each perfusion, hearts were frozen with pre-cooled Wollemburger tongs and stored in liquid nitrogen, until the relevant determinations were carried out.

2.3. Experimental groups

The hearts were randomly divided into:

- Control hearts (n=30), receiving perfusion buffer
- Carvedilol-treated hearts (n=49). On the basis of preliminary dose–response curve studies, carvedilol was used at two concentrations: 0.1 µM, which has no negative inotropic effect, and 2.0 µM, with marked negative inotropic effects
- Propranolol-treated hearts (n=47), receiving two dosages of propranolol, i.e. 1.0 and 5.0 µM, the former lacking a significant negative inotropic effect and the latter showing such an effect

Each treatment was carried out 30 min before ischaemia and throughout the experiment.

2.4. CPK and glutathione release

Coronary effluent was collected in chilled glass vials and assayed, on the same day, for CPK activity. An aliquot of 2.0 ml was added to 0.2 ml of 0.02 M EDTA for immediate GSH and GSSG determination.

CPK was assayed spectrophotometrically by the Oliver method [17] while GSH and GSSG by the method of Tietze [18] as previously described [16].

2.5. Assay of high-energy phosphates, purine and pyridine nucleotides

After each perfusion, the hearts were freeze-clamped with aluminium tongs which had been pre-cooled in liquid nitrogen and stored in liquid nitrogen until the measurements were carried out. High-energy phosphates, purine and pyridine nucleotides were extracted from frozen tissues, both ground (mixed with 0.4 N HClO₄ in liquid nitrogen bath) and homogenised using an Ultra-Turrax, as previously described [19]. Separation and quantification of the specifically extracted metabolites were performed by
HPLC with the use of reversed-phase 3 μm C₁₈ column, as previously described [19]. The mobile phase consisted of a continuous gradient of acetonitrile (2.5–25% v/v) in phosphate buffer from pH 6.0 to 5.5. Detection was performed at 250 nm for creatine phosphate (CP) and at 260 nm for adenylic nucleotides.

Energy charge was calculated as \([\frac{[ATP]+0.5[ADP]}{[ATP]+[ADP]+[AMP]}]\).

2.6. Oxidative stress

A portion of frozen left ventricular apex (~100 mg) was deproteinized with 3 M HClO₄ and the supernatant, obtained after centrifugation at 6000×g for 15 min, neutralised with 2 M K₂CO₃. A sample of the neutralised extract was analysed for total glutathione by the method of Tietze [18]. GSSG was measured as described above, after the preliminary reaction of GSH with 20 mM N-ethylmaleimide followed by complete removal of unreacted sulfhydryl reagent with diethyl ether.

Protein (P-SH) and non protein (NP-SH) –SH were determined as described by Sedlack and Lindsay [20].

2.7. Protein determination

This was carried out according to Bradford [21], using bovine serum albumin as standard.

2.8. Reagents

High grade reagents were obtained from the Sigma Chemical Company (USA). Carvedilol was kindly supplied by Smith Kline & Beecham S.P.A. (Italy).

2.9. Statistical analysis

Data are reported as means±S.E. The statistical significance between means was tested with a two-way ANOVA with linear contrasts.

Spearman’s correlation coefficients among the variables measuring the oxidative stress (expressed as GSH/GSSG after 30 min of reperfusion), diastolic pressure and total CPK release measured during reperfusion (area under the curves), were calculated.

Since our data-sets consisted also of repeated measures, we performed an analysis of variance for repeated measures. With this model, we have analysed the following parameters: systolic pressure, diastolic pressure, CPK, GSH and GSSG releases in relation to time.

The statistical packages SAS (SAS Institute Inc., Cary, NC, USA) was used to perform the statistical analysis. 

\(P\) values <0.05 were considered statistically significant.

3. Results

3.1. Myocardium viability

3.1.1. LV function

Fig. 1 shows a typical functional tracing of each group of experiments both under aerobic and ischaemic conditions. The mean results of the haemodynamic parameters are reported in Fig. 2.

3.1.1.1. Aerobic perfusion studies Aerobic perfusion studies were carried out on hearts perfused for 135 min. The control hearts slightly decreased their CPP (from 41.7±7.2 to 33.1±5.0 mmHg) and were able to maintain developed pressure during aerobic perfusion (92.7±9.9% of the starting value). Under the same conditions, carvedilol at the higher dosage (2.0 μM), but not at 0.1 μM, exerted negative inotropic effects [to 50.8±3.6% (\(P<0.01\)) of baseline developed pressures] (data not shown).

Propranolol at the dose of 5.0 μM exerted a marked negative inotropic action, reaching a developed pressure of 57.7±4.1% vs. baseline (\(P<0.01\)), while at 1.0 μM it did not have any effect (data not shown).

Neither carvedilol nor propranolol affected the CPP at any dosage. Diastolic pressure remained close to 0 mmHg for the entire period of perfusion in each group.

3.1.1.2. Ischaemia and reperfusion studies After 5 min of ischaemia, in control hearts, developed pressure decreased, leading to contractile quiescence. After 30 min of ischaemia, diastolic pressure began to rise up to 42.9±4.3 mmHg. Reperfusion produced a further increase in diastolic pressure (59.7±4.3 mmHg) and a poor recovery in developed pressure (34.1±14.6% from baseline) (Fig. 2A).

Carvedilol at 0.1 μM had no effect on functional parameters during ischaemia. However, it reduced the reperfusion-induced increase in diastolic pressure (from 59.7±4.3 to 36.2±7.8 mmHg, \(P<0.05\)) thus improving the recovery of developed pressure (from 34.1±14.6 to 54.5±9.2%, \(P<0.05\)) (Fig. 2B). In comparison with ischaemic control hearts, carvedilol at 2.0 μM: (a) abolished any rise in diastolic pressure both during ischaemia and reperfusion, (b) increased recovery of developed pressure (97.4±5.0 vs. 34.1±14.6%, \(P<0.01\)), (Fig. 2B), (c) reduced the reperfusion-induced rise of CPP (66.8±9.7 vs. 72.9±5.0 mmHg) (data not shown). Also, 1.0 μM propranolol had no effect on the mechanical function of the isolated heart either during ischaemia or reperfusion. On the contrary, the higher dosage (5.0 μM) preserved mechanical function, although the degree of cardiac protection was smaller than that exerted by carvedilol at the dose of 2.0 μM (Fig. 2C).
Fig. 1. Typical functional tracings relevant to aerobic perfused and ischaemic perfused hearts, untreated and treated with different doses of carvedilol and propranolol.

3.1.2. Membrane damage

The effects on CPK release are in keeping with the results obtained on LV function.

3.1.2.1. Aerobic perfusion studies

The control hearts showed only a minimal amount of CPK release into the coronary effluent (0.44±0.08 mU/min/g wet weight at the
Fig. 2. Mean values of left ventricular (LV) systolic (upper lines) and diastolic (lower lines) pressures in aerobic and ischaemic control hearts (panel A), in 0.1 and 2.0 µM carvedilol hearts (panel B) and in 1.0 and 5.0 µM propranolol hearts (panel C). The drugs were delivered for 30 min before ischaemia and during the experiment.
end of perfusion). Treatment with either carvedilol or propranolol had no effect, independently from the doses (data not shown).

3.1.2.2. Ischaemia and reperfusion studies In control hearts a marked membrane damage was observed. During ischaemia, in view of the severe reduction of the CF, CPK release was not different from aerobic conditions. However, during reperfusion, a progressive and massive CPK release was detected (from $37.8\pm5.9$ to $1816.7\pm200.1$ mU/min/g wet weight (g ww), at 1 and 30 min reperfusion, respectively) (Fig. 3A).

A significant reduction of CPK release during reperfusion was observed with 0.1 µM carvedilol ($1250.6\pm174.6$ vs. $1816.7\pm200.1$ mU/min/g ww, $P<0.05$). Carvedilol at 2.0 µM almost abolished CPK leakage ($309.6\pm19.9$ vs. $1816.7\pm200.1$ mU/min/g ww, $P<0.001$) (Fig. 3A).

On the contrary, the lower dose of propranolol, which lacked β-blocking effects in terms of mechanical function, had no effect on CPK release during reperfusion (1848.3±404.3 vs. 1816.7±200.1 mU/min/g ww) (Fig. 3A). At the higher dosage, propranolol almost abolished the reperfusion-induced CPK release ($P<0.01$).

3.1.3. Cellular energetic status

3.1.3.1. Aerobic perfusion studies As shown in Table 1, 135 min perfusion resulted in a slight, non-significant reduction of ATP and CP myocardial content in control hearts (from $23.7\pm2.1$ to $19.6\pm1.4$ and from $50.4\pm5.2$ to $47.2\pm3.6$ µmol/g dry weight (g dw) (in freshly excised non-perfused and aerobic perfused control hearts, respectively). Neither carvedilol nor propranolol affected these parameters at any dosage.

3.1.3.2. Ischaemia and reperfusion studies A drastic alteration of the cellular energetic status was observed in control hearts. Ischaemia caused a severe depletion of CP and a less severe reduction of ATP. AMP increased proportionally; energy charge decreased from $0.91\pm0.03$ to $0.64\pm0.01$ ($P<0.01$) and the ratio of oxidized and reduced nicotinamide adenine dinucleotide (NAD/NADH) dropped from $8.7\pm0.5$ to $1.0\pm0.1$ ($P<0.01$). Reperfusion caused an additional drop in ATP without any recovery of the other parameters.

Treatment with carvedilol at 0.1 µM had no effect on the metabolic changes induced by ischaemia. However, it increased the recovery of energetic metabolism during reperfusion. In particular, at the end of reperfusion, ATP content was $10.9\pm0.8$ µmol/g dw, $P>0.01$ vs. control and the energy charge was $0.75\pm0.01$, $P<0.01$ vs. control.

The 2.0-µM carvedilol resulted in an important and significant energy saving, during ischaemia: the drop of ATP and CP was significantly reduced; NAD/NADH ratio was also better maintained. As a consequence, energy charge at the end of ischaemia was higher than in control hearts ($0.80\pm0.04$ vs. $0.64\pm0.01$, $P<0.05$). Reperfusion further improved these parameters, suggesting a recovery of the aerobic metabolism.

Propranolol, at the dose of 1.0 µM, failed to improve cardiac metabolism either after ischaemia or reperfusion. The increase of the dose resulted in an improvement similar to that observed with the higher dose of carvedilol.

3.2. Oxidative stress

3.2.1. Aerobic perfusion studies

In both control and treated hearts, with either carvedilol or propranolol, no differences in GSH, GSSG, P- and NP-SH (similar to the results for the other parameters) were observed. The release of GSH and GSSG was negligible and unaffected by the treatment (data not shown).

3.2.2. Ischaemia and reperfusion studies A significant oxidative stress mostly occurring on reperfusion was observed in control hearts. As expected, the rate of GSH and GSSG release was negligible during ischaemia but increased remarkably after reperfusion (Fig. 3A). At the end of ischaemia, GSH/GSSG ratio decreased (from $124.7\pm13.1$ to $41.3\pm6.2$, $P<0.01$; Table 2) due to the fact that GSH content decreased (from $10.9\pm2.0$ to $7.1\pm1.3$ nmol/mg prot, $P<0.05$) and GSSG increased (from $0.09\pm0.02$ to $0.16\pm0.02$ nmol/mg prot, $P<0.01$). After 3 (early) and 30 min (late) reperfusion, there was a further decrease in GSH content ($5.7\pm1.1$ and to $6.2\pm0.5$ nmol/mg prot, respectively; both $P<0.01$ vs. $10.9\pm2.0$ nmol/mg prot observed under aerobic conditions). Conversely, massive accumulation of GSSG was observed at both early and late reperfusion ($0.39\pm0.03$ and $0.31\pm0.01$ nmol/mg prot, respectively, both $P<0.01$ vs. $0.06\pm0.01$ nmol/mg prot observed under aerobic conditions), despite the continuous release, which suggests the occurrence of oxidative stress (Fig. 3C). Consequently, GSH/GSSG ratio further decreased (Table 2). P- and NP-SH decreased from $231.6\pm15.3$ to $122.5\pm8.2$ and from $21.4\pm3.6$ to $10.3\pm1.0$ nmol/mg prot at early reperfusion ($P<0.01$) and to $138.4\pm5.2$ and to $12.3\pm0.7$ nmol/mg prot at late reperfusion ($P<0.01$) (Table 2).

Carvedilol delayed and counteracted the occurrence of oxidative stress during ischaemia and reperfusion. GSH, P- and NP-SH myocardial contents were higher in treated hearts and myocardial GSSG accumulation and release were significantly ($P<0.01$) reduced in a similar pattern in all carvedilol 0.1 and 2.0 µM treated hearts vs. ischaemic control (in early reperfusion: $0.30\pm0.03$ and $0.21\pm0.02$ vs. $0.39\pm0.03$ nmol/mg prot, $P<0.01$; in late reperfusion: $0.25\pm0.02$ and $0.19\pm0.02$ vs. $0.31\pm0.01$; $P<0.01$). In parallel, GSH/GSSG ratio increased (Table 2).

The 5.0-µM propranolol significantly preserved myocardial thiolic group contents and attenuated GSSG accumulation and release, as well. However, the degree of protection
against oxidative stress was less than that obtained by 2.0 μM carvedilol. At 1.0 μM, propranolol showed no improvement, while 0.1 μM carvedilol — which did not affect baseline contractile function — significantly reduced oxidative stress (Fig. 3B,C and Table 2).

3.3. Correlations

We observed a link between carvedilol’s anti-oxidant and cardio-protective effects. In particular, we found a significant correlation between myocardial GSH/GSSG
1. Carvedilol improves myocardium viability in ischaemic and perfused hearts and its effect is better than propranolol.

2. Some of the effects of carvedilol differ from those of propranolol since they are independent from its β-blocking and negative inotropic effect.

3. The anti-oxidant activity of carvedilol may be important in explaining the extra-protection occurring in respect to propranolol.

4.1. Improvement of ischaemic myocardium viability with carvedilol at negative inotropic doses.

In vivo, carvedilol exerts an α₁-adrenergic blocking activity, and, through this mechanism, it may be cardioprotective by unloading the heart. It also reduces heart rate and could, through this mechanism, reduce the myocardial oxygen consumption. However, none of these effects may be considered as having occurred in our experimental model, which was independent from pre- and after-load, and in which the hearts were constantly paced.

In vitro, β-blockers are usually cardioprotective through their negative inotropic effect [22]. In fact, there is an inverse relationship between the degree of contraction before ischaemia and ATP content at the end of ischaemia [23]. This was indeed the case for both carvedilol and propranolol.

**Table 1**

<table>
<thead>
<tr>
<th>ATP (μmol/g dry wt)</th>
<th>Aerobia</th>
<th>Ischaemia</th>
<th>Reperfusion</th>
<th>$P_1$</th>
<th>$P_2$</th>
<th>$P_3$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>19.6±1.4 (6)</td>
<td>6.9±1.1 (6)</td>
<td>7.8±0.4 (12)</td>
<td>0.01</td>
<td>0.01</td>
<td>n.s.</td>
</tr>
<tr>
<td>Carvedilol (0.1 μM)</td>
<td>21.0±2.1 (6)</td>
<td>6.4±1.3 (6)</td>
<td>10.9±0.8 (7)**</td>
<td>0.01</td>
<td>0.01</td>
<td>0.01</td>
</tr>
<tr>
<td>Carvedilol (2.0 μM)</td>
<td>22.5±2.8 (6)</td>
<td>13.6±1.1 (6)**</td>
<td>16.8±2.1 (6)**</td>
<td>0.01</td>
<td>0.05</td>
<td>0.05</td>
</tr>
<tr>
<td>Propranolol (1.0 μM)</td>
<td>19.6±3.0 (6)</td>
<td>6.6±1.0 (6)</td>
<td>6.3±1.1 (6)</td>
<td>0.01</td>
<td>0.01</td>
<td>n.s.</td>
</tr>
<tr>
<td>Propranolol (5.0 μM)</td>
<td>18.7±2.7 (6)</td>
<td>13.3±0.8 (6)**</td>
<td>14.1±1.6 (5)**</td>
<td>0.05</td>
<td>0.05</td>
<td>n.s.</td>
</tr>
</tbody>
</table>

**CP (μmol/g dry wt)**

<table>
<thead>
<tr>
<th>Control</th>
<th>47.2±3.6 (6)</th>
<th>8.1±0.8 (6)</th>
<th>10.8±0.9 (12)</th>
<th>0.01</th>
<th>0.01</th>
<th>0.01</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carvedilol (0.1 μM)</td>
<td>45.8±4.1 (6)</td>
<td>9.0±0.7 (6)</td>
<td>12.6±0.9 (7)**</td>
<td>0.01</td>
<td>0.01</td>
<td>0.01</td>
</tr>
<tr>
<td>Carvedilol (2.0 μM)</td>
<td>46.9±3.6 (6)</td>
<td>14.2±0.8 (6)**</td>
<td>20.5±0.6 (6)**</td>
<td>0.01</td>
<td>0.01</td>
<td>0.01</td>
</tr>
<tr>
<td>Propranolol (1.0 μM)</td>
<td>44.8±5.0 (6)</td>
<td>7.9±0.6 (6)</td>
<td>10.5±1.6 (6)</td>
<td>0.01</td>
<td>0.01</td>
<td>0.05</td>
</tr>
<tr>
<td>Propranolol (5.0 μM)</td>
<td>43.7±4.2 (6)</td>
<td>12.9±0.5 (6)**</td>
<td>18.6±2.0 (5)**</td>
<td>0.01</td>
<td>0.01</td>
<td>0.01</td>
</tr>
</tbody>
</table>

**Energy charge**

<table>
<thead>
<tr>
<th>Control</th>
<th>0.91±0.03 (6)</th>
<th>0.64±0.01 (4)</th>
<th>0.68±0.02 (12)</th>
<th>0.01</th>
<th>0.01</th>
<th>0.01</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carvedilol (0.1 μM)</td>
<td>0.89±0.04 (6)</td>
<td>0.65±0.03 (4)</td>
<td>0.75±0.01 (7)**</td>
<td>0.01</td>
<td>0.01</td>
<td>0.01</td>
</tr>
<tr>
<td>Carvedilol (2.0 μM)</td>
<td>0.89±0.05 (6)</td>
<td>0.80±0.04 (4)*</td>
<td>0.84±0.05 (6)**</td>
<td>n.s.</td>
<td>n.s.</td>
<td>n.s.</td>
</tr>
<tr>
<td>Propranolol (1.0 μM)</td>
<td>0.90±0.06 (6)</td>
<td>0.66±0.04 (4)</td>
<td>0.63±0.04 (6)</td>
<td>0.01</td>
<td>0.01</td>
<td>n.s.</td>
</tr>
<tr>
<td>Propranolol (5.0 μM)</td>
<td>0.90±0.03 (6)</td>
<td>0.78±0.04 (4)*</td>
<td>0.81±0.04 (5)**</td>
<td>0.05</td>
<td>0.05</td>
<td>n.s.</td>
</tr>
</tbody>
</table>

**NADH/NAD**

<table>
<thead>
<tr>
<th>Control</th>
<th>8.7±0.5 (6)</th>
<th>1.0±0.1 (6)</th>
<th>1.2±0.1 (12)</th>
<th>0.01</th>
<th>0.01</th>
<th>0.05</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carvedilol (0.1 μM)</td>
<td>9.1±0.3 (6)</td>
<td>1.1±0.1 (6)</td>
<td>1.3±0.2 (7)</td>
<td>0.01</td>
<td>0.01</td>
<td>n.s.</td>
</tr>
<tr>
<td>Carvedilol (2.0 μM)</td>
<td>9.2±0.5 (6)</td>
<td>2.3±0.1 (6)**</td>
<td>3.1±0.1 (6)**</td>
<td>0.01</td>
<td>0.01</td>
<td>0.01</td>
</tr>
<tr>
<td>Propranolol (1.0 μM)</td>
<td>9.0±0.6 (6)</td>
<td>1.0±0.1 (6)</td>
<td>1.3±0.2 (6)</td>
<td>0.01</td>
<td>0.01</td>
<td>n.s.</td>
</tr>
<tr>
<td>Propranolol (5.0 μM)</td>
<td>8.9±0.5 (6)</td>
<td>2.2±0.1 (6)**</td>
<td>3.0±0.2 (5)**</td>
<td>0.01</td>
<td>0.01</td>
<td>0.01</td>
</tr>
</tbody>
</table>

---

* Data are reported as mean values±S.E. Number of hearts are reported in brackets. $P_1$ indicates the significance of difference between values after aerobia and ischaemia; $P_2$, the significance of difference between values after aerobia and reperfusion; $P_3$, the significance of difference between values after ischaemia and reperfusion; CP, creatine phosphate; ATP, adenosine triphosphate; ADP, adenosine diphosphate; AMP, adenosine monophosphate; energy charge is calculated as $\left[\frac{[\text{ATP}+0.5[\text{ADP}]]}{[\text{ATP}]+[\text{ADP}]+[\text{AMP}]}\right]$; NADH/NAD, ratio of oxidised and reduced nicotinamide adenine dinucleotide.

* $P<0.05$ significant difference vs. control.

** $P<0.01$ significant difference vs. control.

The ratio (at the end of reperfusion) and total CPK release during reperfusion considering both/all groups ($n=34$; $r=-0.674$, $P<0.001$) and only 0.1 μM carvedilol group ($n=7$; $r=-0.782$, $P=0.038$). We obtained similar findings comparing GSH/GSSG ratio with diastolic pressure increase ($r=-0.712$, $P<0.001$ in all groups and $r=-0.730$, $P=0.043$ in 0.1 μM carvedilol).

It is interesting to underline that, in 0.1 μM carvedilol group, no significant correlation was found between GSH/GSSG ratio and ATP ($n=7$; $r=0.570$, $P=0.181$) and CP ($n=7$; $r=0.527$, $P=0.224$) myocardial contents.

4. Discussion

Our data suggest that:

1. Carvedilol improves myocardium viability in ischaemic and perfused hearts and its effect is better than propranolol.

2. Some of the effects of carvedilol differ from those of propranolol since they are independent from its β-blocking and negative inotropic effect.

3. The anti-oxidant activity of carvedilol may be important in explaining the extra-protection occurring in respect to propranolol.
propranolol when used at doses which exerted negative inotropic effects as a result of the β-blockage. In both cases ATP was better maintained at the end of ischaemia. In this condition, readmission of oxygen with reperfusion results in a prompt recovery of intermediate metabolism. This is suggested by the observed re-oxidation of NADH which has been accumulated during ischaemia (Table 1). It is known that both cytosolic and mitochondrial NAD/NADH pools are affected by ischaemia and reperfusion conditions. The method for determining NAD/NADH couple that we used in our study does not distinguish the possible compartmentalisation of pyridinic nucleotides (i.e. mitochondrial vs. cytosolic). However, NAD/NADH measurement in the whole homogenate, mainly represented by the mitochondrial fraction, is a good approximation of the actual status of the Krebs cycle [24,25]. In turn, the decrease of cellular accumulation of NADH, putatively due to re-established electron-flow through respiratory chain, leads to increased tissue contents of high-energy phosphates.

A reduced energy breakdown during ischaemia is expected to maintain cellular viability, thus avoiding membrane damage, alteration of ionic homeostasis and the occurrence of oxidative stress. Therefore, the possible effects on glutathione and -SH group metabolism which we found after treatment with high doses of carvedilol and propranolol are most likely the consequence of a generalised cardiac protection due to the β-blocking-mediated energy saving.

Table 2

<table>
<thead>
<tr>
<th></th>
<th>Aerobia</th>
<th>Ischaemia</th>
<th>Early (3 min) reperfusion</th>
<th>Late (30 min) reperfusion</th>
<th>$P_1$</th>
<th>$P_2$</th>
<th>$P_3$</th>
<th>$P_4$</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>GSH/GSSG</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>124.7±13.1 (6)</td>
<td>41.3±6.2 (6)</td>
<td>14.6±2.0 (6)</td>
<td>20.3±1.8 (12)</td>
<td>0.01</td>
<td>0.01</td>
<td>0.01</td>
<td>n.s.</td>
</tr>
<tr>
<td>Carvedilol (0.1 μM)</td>
<td>143.1±110 (6)</td>
<td>101.2±5.8 (6)**</td>
<td>21.7±3.1 (6)</td>
<td>28.7±2.1 (7)**</td>
<td>0.05</td>
<td>0.01</td>
<td>0.01</td>
<td>n.s.</td>
</tr>
<tr>
<td>Carvedilol (2.0 μM)</td>
<td>127.8±162 (6)</td>
<td>135.1±8.1 (6)**</td>
<td>38.0±2.7 (6)**</td>
<td>47.4±3.1 (6)**</td>
<td>n.s.</td>
<td>0.01</td>
<td>0.01</td>
<td>n.s.</td>
</tr>
<tr>
<td>Propranolol (1.0 μM)</td>
<td>133.5±10.2 (6)</td>
<td>45.2±5.6 (6)</td>
<td>14.3±1.9 (6)</td>
<td>18.1±3.3 (6)</td>
<td>0.01</td>
<td>0.01</td>
<td>0.01</td>
<td>n.s.</td>
</tr>
<tr>
<td>Propranolol (5.0 μM)</td>
<td>114.3±10.7 (6)</td>
<td>73.1±9.1 (6)*</td>
<td>22.2±3.0 (6)*</td>
<td>29.4±3.4 (5)*</td>
<td>0.05</td>
<td>0.01</td>
<td>0.01</td>
<td>n.s.</td>
</tr>
<tr>
<td><strong>P-SH (nmol/mg protein)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>231.6±15.3 (6)</td>
<td>190.2±18.1 (6)</td>
<td>122.5±8.2 (6)</td>
<td>138.4±5.2 (12)</td>
<td>0.05</td>
<td>0.01</td>
<td>0.01</td>
<td>0.01</td>
</tr>
<tr>
<td>Carvedilol (0.1 μM)</td>
<td>224.5±18.2 (6)</td>
<td>210.7±13.4 (6)</td>
<td>130.3±13.2 (6)</td>
<td>151.9±7.5 (7)</td>
<td>n.s.</td>
<td>0.01</td>
<td>0.01</td>
<td>0.01</td>
</tr>
<tr>
<td>Carvedilol (2.0 μM)</td>
<td>232.6±15.1 (6)</td>
<td>219.6±17.3 (6)</td>
<td>145.8±13.6 (6)*</td>
<td>169.3±5.3 (6)**</td>
<td>n.s.</td>
<td>0.01</td>
<td>0.01</td>
<td>0.05</td>
</tr>
<tr>
<td>Propranolol (1.0 μM)</td>
<td>235.8±16.6 (6)</td>
<td>200.6±14.5 (6)</td>
<td>121.5±10.9 (6)</td>
<td>132.4±14.6 (6)</td>
<td>0.05</td>
<td>0.01</td>
<td>0.01</td>
<td>n.s.</td>
</tr>
<tr>
<td>Propranolol (5.0 μM)</td>
<td>217.6±12.2 (6)</td>
<td>195.2±17.1 (6)</td>
<td>157.4±21.4 (6)*</td>
<td>180.7±27.6 (5)**</td>
<td>n.s.</td>
<td>0.01</td>
<td>0.01</td>
<td>n.s.</td>
</tr>
<tr>
<td><strong>NP-SH (nmol/mg protein)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>21.4±3.6 (6)</td>
<td>14.3±4.0 (6)</td>
<td>10.3±1.0 (6)</td>
<td>12.3±0.7 (12)</td>
<td>n.s.</td>
<td>0.01</td>
<td>0.01</td>
<td>n.s.</td>
</tr>
<tr>
<td>Carvedilol (0.1 μM)</td>
<td>21.5±2.9 (6)</td>
<td>17.8±3.3 (6)</td>
<td>12.6±1.6 (6)</td>
<td>14.4±1.0 (7)**</td>
<td>n.s.</td>
<td>0.01</td>
<td>0.01</td>
<td>n.s.</td>
</tr>
<tr>
<td>Carvedilol (2.0 μM)</td>
<td>22.3±2.5 (6)</td>
<td>18.9±3.0 (6)</td>
<td>14.5±2.2 (6)*</td>
<td>17.9±0.7 (6)**</td>
<td>n.s.</td>
<td>0.01</td>
<td>0.05</td>
<td>0.05</td>
</tr>
<tr>
<td>Propranolol (1.0 μM)</td>
<td>20.3±1.8 (6)</td>
<td>15.4±2.4 (6)</td>
<td>8.8±1.3 (6)</td>
<td>10.8±2.0 (6)</td>
<td>0.05</td>
<td>0.01</td>
<td>0.01</td>
<td>n.s.</td>
</tr>
<tr>
<td>Propranolol (5.0 μM)</td>
<td>22.8±2.0 (6)</td>
<td>17.9±3.1 (6)</td>
<td>13.4±1.9 (6)</td>
<td>14.4±1.8 (5)*</td>
<td>0.05</td>
<td>0.01</td>
<td>0.05</td>
<td>n.s.</td>
</tr>
</tbody>
</table>

* Data are reported as mean values±S.E. Number of hearts are reported in brackets. $P_1$ indicates the significance of difference between values after aerobia and ischaemia, $P_2$, the significance of difference between values after aerobia and early reperfusion; $P_3$, the significance of difference between values after aerobia and late reperfusion; $P_4$, the significance of difference between values after early and late reperfusion; GSH, reduced glutathione; GSSG, oxidised glutathione; P-SH, protein sulphhydryl groups; NP-SH, non-protein sulphhydryl groups.

* Indicates $P<0.05$ significant difference vs. control.

** $P<0.01$ significant difference vs. control.

When used at 0.1 μM, carvedilol had no effect on inotropism or on energy metabolism and actually maintained myocardium viability and recovery on reperfusion. This was not the case for propranolol. Thus, the issue is: ‘could this effect of carvedilol, as well as its extra-protection in respect to propranolol, be a consequence of its anti-oxidant activity?’ Actually, our data support this hypothesis.

We measured oxidative stress in terms of glutathione metabolism. Glutathione system plays a crucial role in the myocyte: GSH works as a reducing equivalent donor,
auto-oxidising to GSSG which is reconverted into the reduced form at the expense of NADPH via glutathione reductase. Oxidant conditions, such as post-ischaemic reperfusion, both in humans and in experimental models, induce accumulation of GSSG and reduction of cellular thiol groups in the myocardium [26–29]. Thus, myocardial GSSG content and release are reliable and specific indices of oxidative stress suitable to test the anti-oxidant capacity of a drug [16,30–32].

In our experiments, 0.1 μM carvedilol showed its cardiac beneficial effect mainly at early reperfusion condition (Figs. 2 and 3 and Tables 1 and 2), in which a burst of oxygen free radical production occurs and oxidative stress causes functional alterations [24–27]. After ischaemia, 0.1 μM carvedilol had no effect on energy status nor on intermediate metabolism, as suggested by the finding that NAD/NADH ratio was unaffected. Conversely, the only effect was the reduction of GSH depletion and GSSG accumulation (with parallel reduction in GSH/GSSG) in myocardium tissue (Table 2). During reperfusion, there was a correlation between the indices of viability (i.e. CPK release and LV dysfunction) and GSSG accumulation, but not with high energy content. This suggests a link between oxidative stress and viability, although it is always difficult to draw a clear cause–effect relationship since carvedilol and propranolol have widely disparate pharmacological properties which could also be involved.

In vitro models, carvedilol, through the carbazol group in its moiety, scavenged the hydroxyl radicals and the superoxide anions with IC50 values of 25 and 28 μM, respectively, and, more potently, reduced the lipid peroxidative injury (IC50 of 94.1 nM to 8.1 μM) [4], which is likely to occur on reperfusion, resulting in membrane damage and alteration of ionic homeostasis with consequent CPK release, increase in diastolic pressure and imbalance of systolic function. The dosages of carvedilol used in our experiments are lower than IC50 values for its anti-oxidant activities [4,5,14]. However, this does not invalidate the anti-oxidant mechanism in our experimental setting. The high lipophilicity of its moiety may favour carvedilol accumulation in the myocardial tissue particularly during ischaemia when the coronary flow is severely reduced thus reaching concentrations severals times higher than those in the perfusion buffer [4], favoured from the long exposure to the drug before, during and after ischaemia, as was applied by ourselves.

5. Conclusions

Differing from previous studies which were performed using doses of carvedilol exerting strong negative inotropic effect, our data show that carvedilol improves ischaemic myocardium viability even when used at non-negative inotropic doses and, thus, suggests that this can be a result of its anti-oxidant activity. Obviously, the above mechanism cannot be accepted tout court, since other factors can intervene in this effect. However, in our in vitro model, alternative mechanisms are difficult to identify since all the variables were kept under control. The actual relevance of carvedilol’s anti-oxidant activity in cardioprotection should be investigated in more complex in vivo models.

Acknowledgements

We thank Roberta Ardesi and Patrizia Martina for technical assistance and Dr Alessandro Bettini and Sandra Marini for editing the manuscript.

References