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Carvedilol inhibits mitochondrial complex I and induces resistance to H_2O_2-mediated oxidative insult in H9C2 myocardial cells

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Abstract

Carvedilol, a β-adrenoreceptor antagonist with strong antioxidant activity, produces a high degree of cardioprotection in a variety of experimental models of ischemic cardiac injury. Although growing evidences suggest specific effects on mitochondrial metabolism, how carvedilol would exert its overall activity has not been completely disclosed. In the present work we have investigated the impact of carvedilol-treatment on mitochondrial bioenergetic functions and ROS metabolism in H9C2 cells. This analysis has revealed a dose-dependent decrease in respiratory fluxes by NAD-dependent substrates associated with a consistent decline of mitochondrial complex I activity. These changes were associated with an increase in mitochondrial H_2O_2 production, total glutathione and protein thiols content. To evaluate the antioxidant activity of carvedilol, the effect of the exposure of control and carvedilol-pretreated H9C2 cells to H_2O_2 were investigated. The H_2O_2-mediated oxidative insult resulted in a significant decrease of mitochondrial respiration, glutathione and protein thiol content and in an increased level of GSSG. These changes were prevented by carvedilol-pretreatment. A similar protective effect on mitochondrial respiration could be obtained by pre-treatment of the cells with a sub-saturating amount of rotenone, a complex I inhibitor.

We therefore suggest that carvedilol exerts its protective antioxidant action both by a direct antioxidant effect and by a preconditioning-like mechanism, *via* inhibition of mitochondrial complex I.

Keywords: Mitochondria; Respiratory chain; Complex I; Carvedilol; Oxidative stress; Glutathione

1. Introduction

Cardiovascular diseases represent a leading cause of death worldwide. Considerable evidence suggests that oxidative stress is intimately involved in the pathogenesis of various forms of cardiovascular diseases, including myocardial ischemia–reperfusion injury, congestive heart failure, arteriosclerosis, and drug-induced cardiomyopathy [1–4]. In support to these notions, administration of exogenous antioxidant agents has been shown to result in protective effects on oxidative myocardial injury [5,6].

Carvedilol, a non-selective β-blocker with both β_1- and β_2-adrenoreceptor and α_1-receptor blocking properties, is widely used as anti-hypertensive medicament [7] and improves cardiac function in patients with heart failure by decreasing heart rate, myocardial contractility and myocardial oxygen demand [8–10]. Some of the carvedilol effects have been assigned to its antioxidant action [6,11,12] which might contribute to the higher cardioprotection afforded by carvedilol as compared with other β-blockers [13,14]. Carvedilol has been shown (i) to prevent hydroxyl radicals-induced decrease in sarcoplasmatic reticulum Ca^{2+}-ATPase activity [15], (ii) to attenuate the hydrogen peroxide (H_2O_2)-mediated decrease of mRNA for Ca^{2+}-ATPase by enhancing gene transcription for this enzyme [16], (iii) to exert protection against oxidative damages induced by ischemia–reperfusion [17] and by the ROS-producing hypoxanthine/xanthine oxidase activated system [18,19].

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However, the mechanism by which carvedilol would exert its overall activity has not been completely disclosed and several hypotheses have been proposed.

An interesting aspect of the carvedilol action is related to its specific effect on mitochondrial metabolism. Although the primary role of mitochondria is the ATP generation through oxidative phosphorylation (OXPHOS), these organelles are also involved in the initiation and execution of apoptotic cell death and in the calcium and iron homeostasis [20]. Furthermore, the electron transfer along the mitochondrial electron transport complexes can be associated with single electron reduction of molecular oxygen to form superoxide anions. It has been estimated that as much as 1–2% of the overall oxygen consumption can result in the formation of reactive oxygen species (ROS), with the majority being generated by mitochondria [21].

Evidence has emerged that energy deficiency can have a causative link with heart failure, but, as well, it may be adaptively regulated under certain conditions. For example, in the ischemic heart, a reduction in the mitochondrial oxidative capacity serves to reduce oxygen consumption in response to the limited O_2 availability. Therefore, drugs able to modulate mitochondrial functions might have new and unexpected development in the clinical practice and, in particular, in patients with cardiac disease.

Carvedilol has been shown to prevent, in isolated heart mitochondria, the calcium-induced mitochondrial permeability transition (MPT) occurring in the post-ischemic reperfusion of the heart [22] by avoiding protein thiol oxidation [23]. It also improves mitochondrial energy production, thus decreasing the oxidative phosphorylation lag phase during ischemia [10]. On the other hand, carvedilol can exert pro-oxidant effects. In fact, it has been reported that this drug can induce, in isolated mitochondria, a marked inhibition of mitochondrial respiration by NAD-dependent substrates [24] and a specific inhibition of mitochondrial complex I activity [24,25], with these effects being associated with an increased production of ROS.

In the first part of the present work we have investigated the effects of carvedilol on mitochondrial respiratory fluxes, OXPHOS enzymatic activities and oxidative stress markers in H9C2 cells. The most important observation obtained by this analysis is the dose-dependent decrease of respiratory fluxes by NAD-dependent substrates and the parallel decline of complex I activity. These effects were associated with an increase in mitochondrial H_2O_2 production and in total glutathione and protein thiols content. We have then studied the antioxidant properties of the drug by applying a pro-oxidant insult (H_2O_2) on carvedilol pre-treated cells. The results show that carvedilol pre-treatment induces a significant protection from subsequent oxidative damage and suggest the possibility of a preconditioning-like mechanism of action besides a direct antioxidant effect of the drug.

2. Materials and methods

2.1. Cell lines and media

The H9C2 cell line, derived from embryonic rat heart [26], was purchased from American Type Culture Collection (ATCC; Virginia, USA). Cells were cultured, without tubular structure formation, in high-glucose Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) foetal bovine serum (FBS), 1% (v/v) L-glutamine, 1% (v/v) penicillin/streptomycin, at 37 °C in a humidified atmosphere of 10% CO_2. Cells were plated and subcultured at 70–80% of confluence according to ATCC Product information sheet. Cell viability was assessed by trypan blue exclusion test.

2.2. Carvedilol treatment

Carvedilol, kindly supplied by GlaxoSmithKline (West Sussex, UK), was dissolved in dimethyl sulfoxide (DMSO) and added to the cells at 60–70% of confluence, at the indicated final concentrations. Cells treated in parallel with equal amounts of DMSO were used as control cells. However, the maximal DMSO concentration reached in our experiments (about 0.05%) did not cause any detectable effect on cell viability and/or on any other parameter measured in the present work. No change in respiration rate was observed in time 0 control as well as upon direct addition of carvedilol into the oxygraphic chamber both to control intact cells and to control permeabilized cells respiring on complex I or complex II substrates. A time response curve of cellular respiration at 10 μM carvedilol indicated that the maximal inhibitory effect of respiratory activity was reached at 12 h and remained fairly constant up to 24–28 h of treatment (data not shown). We therefore decided to set the standard incubation time with carvedilol at 24 h. Under these conditions, no effect on cell viability could be detected. In order to investigate the protective effect of carvedilol from oxidative insults, control cells or carvedilol (10 μM)-pretreated cells, were exposed, for additional 2 h, to increasing concentration of H_2O_2, upon removal of DMSO- or carvedilol-containing medium, respectively. The same conditions were used to investigate the effects of rotenone (0.1 nM)-pretreatment on H_2O_2 (200 μM)-mediated oxidative insult.

2.3. Measurement of endogenous respiration rates in intact cells and substrate-supported respiration rates in digitonin-permeabilized cells

The respiratory activity was measured polarographically with a Clark-type oxygen electrode in a water-jacketed chamber, (Hansatech Instruments, Norfolk, UK), magnetically stirred at 37 °C essentially as previously described [27]. Cells, fluid changed 1 day before the measurement, were collected by trypsinization and centrifugation and resuspended at $1–3\times10^6$ cells/ml in TD Buffer (0.137 M NaCl, 5 mM KCl, 0.7 mM Na_2HPO_4, 25 mM Tris–HCl, pH 7.4), for measurements of mitochondrial respiratory fluxes, or in Buffer A (75 mM sucrose, 5 mM KH_2PO_4, 40 mM KCl, 0.5 mM EDTA, 3 mM $MgCl_2$, 30 mM Tris–HCl, pH 7.4), supplied with 0.3 mM P1,P5-di(adenosine-5′) pentaphosphate (Ap5A) to prevent dissipation of ADP by adenylate kinase, for the measurements of OXPHOS efficiency (P/O ratios). The cell suspension was transferred to the polarographic chamber and an aliquot was utilized for cell counting and protein determination. After permeabilization by digitonin, where indicated, substrates and inhibitors of mitochondrial OXPHOS were added at the following concentrations: pyruvate (5 mM)/malate (2.5 mM), succinate (5 mM), ascorbate (10 mM) + TMPD (0.4 mM), rotenone (200 nM), antimycin A (15 nM) and cyanide (1 mM). In the experiments for OXPHOS efficiency measurement, the substrates pyruvate/malate and succinate were followed by addition of 0.17 mM ADP and 0.08 mM ADP, respectively, to induce transient stimulation of oxygen consumption.

2.4. Determination of mitochondrial enzyme activities

Cells, collected by trypsinization and centrifugation, were resuspended in buffer A, as described above, counted and supplemented with 30 μg/10^6 cells of digitonin. After 1–2 min, the cell suspension was further diluted by adding 9 volumes of the same buffer and centrifuged; the pellet was then resuspended in hypotonic medium (25 mM potassium phosphate, pH 7.2, 5 mM $MgCl_2$), supplemented with antiproteases cocktail tablet (Boehringer Mannheim). In order to allow complete accessibility of substrates to the inner mitochondrial membrane enzymes, samples were freeze–thawed three times, gently shaken and then resuspended in the assay buffer described above. All enzyme activities were measured spectrophotometrically with a Beckman DU7400 equipped with a rapid-mixing apparatus at 30 °C essentially as previously described [28] with some minor modifications.

NADH-CoQ oxidoreductase (complex I) activity was assayed in the assay buffer described above supplemented with fatty-acid free bovine serum albumin (BSA) (2.5 mg/ml) in the presence of 2 mM KCN, antimycin A (3 μg/ml) and 65 μM decylubiquinone, using 250 μg/ml of protein, by following the rotenone-sensitive initial rate of NADH oxidation at 340–425 nm ($\Delta\varepsilon = 6.81\ mM^{-1}\cdot cm^{-1}$). Rotenone (2 μg/ml) inhibited the enzymatic activity by 75–80%.

Succinate-cytochrome *c* oxidoreductase (complex II+III) activity was measured at 550–540 nm ($\Delta\varepsilon = 19.1\ mM^{-1}\cdot cm^{-1}$) as initial rate of antimycin-sensitive cytochrome *c* reduction. Prior to the measurement, proteins (130 μg/ml) were incubated for 10 min in the assay buffer in the presence of 20 mM succinate. Rotenone (3 μg/ml), 2 mM KCN and 65 μM decylubiquinone were added to the assay buffer and after baseline recording, the reaction was started by the addition of 20 μM ferricytochrome *c*. The activity was 90–95% inhibited by antimycin A (2 μg/ml).

Cytochrome c oxidase (complex IV) activity was measured by following the oxidation of ferrocytochrome *c* at 550–540 nm ($\Delta\varepsilon = 19.1\ mM^{-1}\cdot cm^{-1}$). Enzymatic activity was estimated in the assay buffer supplemented with 500 μM dodecyl maltoside in the presence of antimycin A (3 μg/ml), using 80 μg/ml of protein, as the apparent first-order rate constant after all cytochrome *c* oxidation induced by the addition of few grains of potassium hexacyanoferrate. This rate was inhibited over 95% by KCN (2 mM).

Citrate synthase activity was used as mitochondrial matrix enzymatic marker. Mitochondrial proteins (80 μg/ml), 0.25 mM acetyl-coenzyme A, and 0.2 mM DTNB were added to a Tris–HCl buffer (10 mM), pH 7.4, containing 0.2% (v/v) Triton X-100. The reaction was started by the addition of 0.4 mM oxalacetate and the initial rate was measured following the reduction of DTNB at 412–360 nm ($\Delta\varepsilon = 13.6\ mM^{-1}\cdot cm^{-1}$).

Protein concentration was determined according to Bradford method, using BSA as standard.

2.5. Free radical production assay

ROS production was measured using the cell permeant probe 2′–7′ dichlorodihydrofluorescin diacetate (DCFDA) which passively diffuses into cells where intracellular esterases cleave the acetate groups to form the impermeable $DCFH_2$ which remains trapped within the cell [29]. After 24-h incubation with carvedilol, cells were collected by trypsinization and resuspended in a small volume of phosphate-buffered saline (PBS) medium. 3×10^5 cells were then incubated in 800 μl of Buffer A with 5 μM DCFDA for 10 min in the dark at 37 °C and the linear fluorescence increase (507 nm excitation and 530 nm emission wavelength) caused by the ROS-dependent oxidation of $DCFH_2$ to the fluorescent compound dichlorofluorescein (DCF) was measured with a Jasco FP6200 spectrofluorimeter.

2.6. Measurement of total (GSH) and oxidized (GSSG) glutathione and of protein sulfhydryl groups (PSH)

Cells, collected by trypsinization and centrifugation, were resuspended in hypotonic medium (25 mM potassium phosphate, pH 7.2, 5 mM $MgCl_2$), supplemented with antiproteases cocktail tablet (Boehringer Mannheim) at a protein concentration of 1.5–2.0 mg protein/ml and homogenized with a glass/glass potter. For total glutathione (GSH) and oxidized fraction of glutathione (GSSG) measurement, proteins were precipitated with 25% sulfosalicylic acid (SSA) (w/v) and separated by centrifugation at 12,000×*g* for 3 min. The supernatant was analyzed for GSH by using the GSSG reductase 5,5-dithiobis-nitrobenzoic acid (DTNB) recycling procedure [30]. For the determination of GSSG, the SSA supernatant was incubated for 1 h after careful addition of 2-vinylpyridine and triethanolamine. Absorbance was measured at 412 nm by using the GSSG reductase recycling procedure [30]. For protein sulfhydryl groups (PSH) measurement, proteins were precipitated with 4% SSA (w/v) and centrifuged at 10,000×*g* for 5 min. The resulting pellet was washed twice with 2% SSA and resuspended in 800 μl of 6 M guanidine. Absorbance was measured at 412 and 530 nm before and 30 min after incubation with 50 μl of 10 mM DTNB [30]. Protein concentration in guanidine dissolved samples was measured by using a Bio-Rad kit for the assay of proteins.

2.7. Statistical analysis

Data are expressed as means ± standard deviation (SD) and were statistically analyzed by the Student's *t* test.

3. Results

3.1. Effect of carvedilol on mitochondrial respiratory capacities

To investigate the effects of carvedilol on mitochondrial functions in H9C2 cardiomyocytes, we have optimized a method [27] for the analysis of respiratory fluxes and OXPHOS efficiency in intact and digitonin-permeabilized cells. In Fig. 1A it is reported a typical polarographic tracing of a standard experiment for the measurement of respiratory capacities. About 2×10^6 cells/ml were suspended in TD Buffer and, after recording the endogenous respiration rate, the maximal endogenous respiratory capacity was measured by adding the uncoupler dinitrophenol (DNP) at a final concentration of 40 μM. This concentration was chosen from preliminary

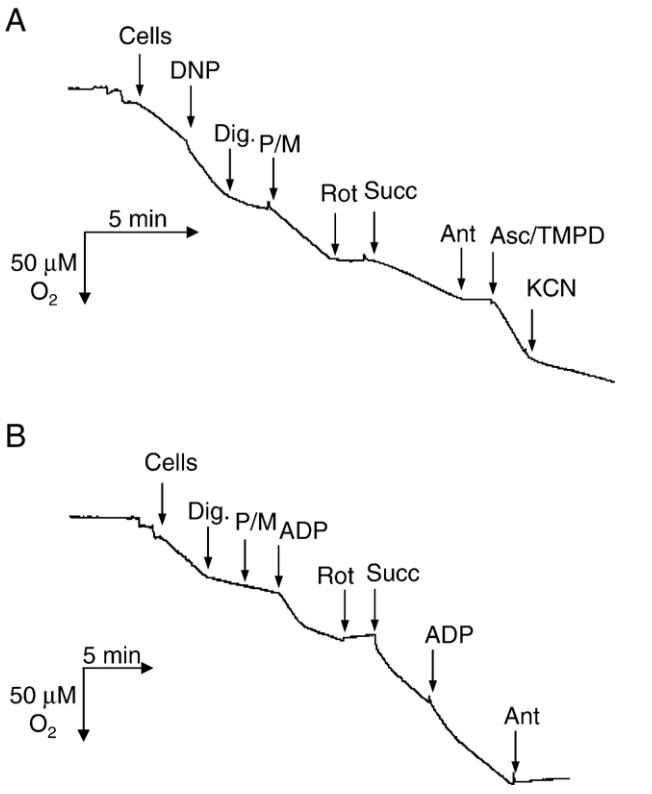

Fig. 1. Measurements of respiration rates in H9C2 cells. (A) Typical polarographic recording of basal and DNP-uncoupled respiration rates by endogenous substrates in intact cells and of exogenous substrate-dependent respiration rates in digitonin-permeabilized cells. (B) Typical polarographic recording of ADP-stimulated respiration rates by exogenous substrates. Cells, H9C2 cells; DNP, dinitrophenol (40 μM); Dig., digitonin (30 μg/10^6 cells); P/M, pyruvate (5 mM)/malate (2.5 mM); ADP (0.17 and 0.08 mM for P/M and Succ, respectively); Rot, rotenone (200 nM); Succ, succinate (5 mM); Ant.A, antimycin A (15 nM); Asc/TMPD, ascorbate (10 mM)+TMPD (0.4 mM); KCN, potassium-cyanide (1 mM).

experiments as the minimal one producing the highest stimulation of the respiratory rate (data not shown) [27]. Afterwards, digitonin was added as permeabilizing agent at a final concentration of 30 μg/10^6 cells. Also in this case, a preliminary digitonin titration was carried out in order to choose the optimal detergent concentration corresponding to the minimal one eliciting the highest respiration rate by the poorly membrane-permeant succinate [27] (data not shown). Following digitonin incubation (2 min), the maximal DNP-uncoupled respiratory fluxes by pyruvate/malate, (complex I+III+IV), succinate, (complex II+III+IV) and ascorbate+TMPD (complex IV) were measured as rates sensitive to inhibition by rotenone, antimycin A and cyanide, respectively.

As shown in Fig. 1B, for the measurements of OXPHOS efficiency (P/O ratios), ~3 ×10^6 cells were resuspended in the assay buffer (Buffer A) and, after permeabilization by digitonin, pyruvate/malate and succinate (+rotenone) were added as respiratory substrates followed by a pulse of subsaturating concentrations of ADP, to induce transient stimulation of the oxygen consumption rate (state 3 respiration). Upon exhaustion of the added ADP the state 4 respiration rate was recorded. The P/O ratio and the respiratory control ratio (RCR) could be therefore calculated as the ratio between the amount (nanomoles) of added ADP and the oxygen (natoms) consumed during the ADP-induced state 3 respiration and as the ratio between state 3 and state 4 respiration rates, respectively.

The effects of carvedilol-treatment of heart-derived H9C2 cells on the basal and the DNP-uncoupled respiration rates by endogenous substrates are shown in Fig. 2. A dose-dependent decrease was observed for both respiratory fluxes. The most significant difference was detected in the DNP-uncoupled endogenous respiration rate which was inhibited by 32% ($p<0.005$, $n=8$) in the presence of 20 μM carvedilol. The data also reveal a dose-dependent decrease by carvedilol in the stimulatory effect of the uncoupler DNP on the endogenous respiration rates.

In order to understand whether the observed decrease of the respiratory fluxes was due to the impairment of specific steps of

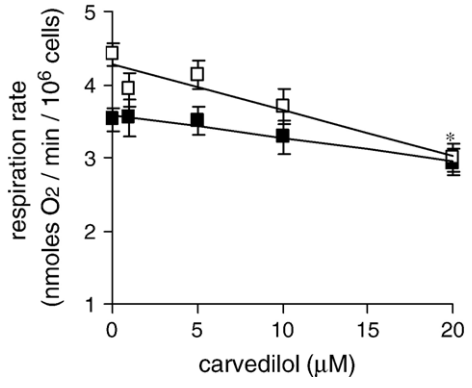

Fig. 2. Effect of carvedilol on endogenous respiration rates in intact cells. H9C2 cells were treated with carvedilol at the indicated concentration for 24 h before measurement of cell respiration. Oxygen consumption rates were measured polarographically in the absence (■) and in the presence (□) of the uncoupler DNP. The data represent the means±SD from eight independent experiments. For experimental details, see under Materials and methods. *$p<0.01$ carvedilol-treated cells versus control cells for DNP-endogenous respiration measurement.

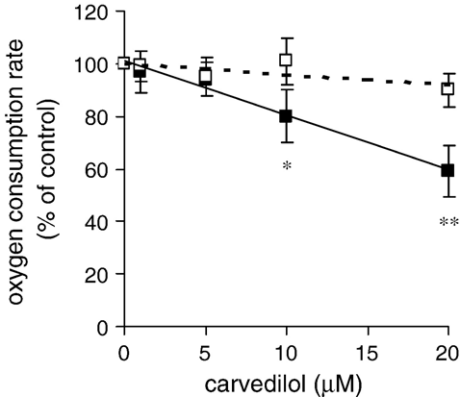

Fig. 3. Effect of carvedilol on maximal mitochondrial respiratory capacities in digitonin-permeabilized cells. H9C2 cells were treated with carvedilol at the indicated concentrations for 24 h before measurement of cell respiration. Oxygen consumption rates, expressed as percentage of control cell values, were measured polarographically in the presence of DNP using pyruvate/malate (100%=3.19±0.15 nmol O_2 /min/10^6 cells) (■) or succinate (100%=2.67±0.11 nmol O_2 /min/10^6 cells) (□) as exogenous respiratory substrates in digitonin-permeabilized cells. The data represent the means±SD from eight independent experiments. For experimental conditions, see under Materials and methods. *$p<0.05$; **$p<0.01$ carvedilol-treated cells versus control cells.

the respiratory chain, the effect of carvedilol on the maximal oxygen consumption rates (in the presence of DNP) by exogenous substrates was measured in digitonin-permeabilized cells as shown in Fig. 1A. The results of this analysis are reported in Fig. 3 and show a marked dose-dependent decrease of mitochondrial uncoupled respiration by NAD-dependent substrates, but not by succinate. In fact, the uncoupled-respiration rates by pyruvate/malate, were decreased by 31% ($p<0.05$, $n=8$) and 41% ($p<0.01$, $n=8$) in the presence of 10 and 20 μM carvedilol, respectively. These data are consistent with the above described decrease in the DNP-uncoupled respiration rates by endogenous substrates (Fig. 2). Similar inhibitory effects were observed when analyzing the respiratory fluxes in phosphorylating conditions, i.e. in the presence of ADP (data not shown). The efficiency of oxidative phosphorylation, measured as coupling of mitochondrial respiration to ATP production, namely as P/O ratio, showed no change at any carvedilol concentration, as compared with control cells, either with pyruvate/malate (P/O=2.53±0.09) or succinate (P/O=1.38±0.05) as respiratory substrates (data not shown).

3.2. Effect of carvedilol on OXPHOS enzymes and citrate synthase activities

To evaluate whether the observed decline of the respiratory fluxes was due to the alteration of specific mitochondrial electron transfer complexes, the enzymatic activities of complex I, complex II+III and complex IV were measured in digitonin-permeabilized cell lysates. The data are shown in Table 1 and reveal a highly significant inhibition by carvedilol of the rotenone-sensitive NADH-CoQ oxidoreductase (complex I) activity which decreased by 25%, 27% and 48% in the presence of 5, 10 and 20 μM carvedilol, respectively. By contrast, the succinate-cytochrome *c* oxidoreductase (complex II+III) and

Table 1
Effect of carvedilol on mitochondrial enzyme activities in H9C2 cells

Enzyme activities (nmol/min/mg protein)	I	II+III	IV	Citrate synthase
Carvedilol (μM)				
0	16.1±1.54	14.8±1.68	250.4±25.5	78.45±7.91
1	14.6±1.71	15.3±1.55	254.5±28.9	77.69±4.69
5	*12.1±1.51	14.1±0.91	245.7±17.9	70.61±7.70
10	*11.7±1.32	13.5±1.11	244.8±22.9	71.21±9.19
20	**8.4±1.28	13.8±1.15	217.7±15.7	73.72±9.58

Mitochondrial enzyme activities were measured on permeabilized-cell lysates as described in Materials and methods. Values are mean±SD from six different experiments and the significance of differences was analyzed by Student's *t* test analysis. *$p<0.05$; **$p<0.001$ carvedilol-treated cells versus control cells.

the cytochrome *c* oxidase (complex IV) activities were unaffected. The mitochondrial enzyme activities expressed as relative to citrate synthase, a mitochondrial matrix marker which was unchanged in carvedilol-treated cells, did show the same pattern of variations so far described.

3.3. Effect of carvedilol on mitochondrial ROS generation, GSH and PSH content

A substantial increase in the rate of ROS generation associated with inhibition of complex I activity by carvedilol has been previously described in isolated mitochondria [24]. In order to assess whether a similar effect could occur at cellular level, measurement of steady-state production of oxygen radicals has been carried out in carvedilol-treated intact cells. ROS production was measured either in basal condition or in the presence of exogenous permeable NAD-dependent substrates. The data obtained are reported in Fig. 4 and show a dose-dependent increase in the ROS production rate, for both conditions, with the most significant variations being detected at 10 and 20 μM carvedilol.

To evaluate the impact of carvedilol-treatment on the oxidative state of the H9C2 cells, the cellular contents of total glutathione (GSH), of oxidized fraction of glutathione (GSSG) and of protein sulfhydryl groups (PSH) were measured. Incubation of cells with carvedilol resulted in a slight but nevertheless highly significant variation of GSH and PSH content, which were increased up to 16% ($p<0.01$, $n=6$) and 22% ($p<0.01$, $n=6$) respectively, as compared with control cells, already at 5 μM carvedilol without any further change at higher concentrations. Conversely, the GSSG content was not significantly changed by carvedilol at any of the utilized concentrations (data not shown).

3.4. Protective effects of carvedilol-pretreatment on H_2O_2-induced cell injury

To test the antioxidant properties of carvedilol in our cellular system, H9C2 cells were pre-treated with 10 μM carvedilol for 24 h and then exposed to H_2O_2 in order to mimic a strong oxidative insult [31].

In a preliminary experiment two different concentrations of H_2O_2 were tested for their effect on cell morphology. As shown in Fig. 5, no evident differences were observed by phase-contrast microscopy between control (Fig. 5A) and carvedilol-treated cells (Fig. 5B) after exposure to 200 μM H_2O_2 for 2 h (Figs. 5C and D, respectively). On the other hand, marked cell shrinkage and membrane blebbing could be observed in H9C2 cells (48%±11% of total cells as counted in five different fields from three independent experiments) as early as 30 min after exposure to a higher (500 μM) H_2O_2 concentration in control cells (Fig. 5E), but not in carvedilol pre-treated cells (Fig. 5F). Based on these observations (see also Ref. [31]), a range of sublethal doses, as monitored by trypan blue exclusion test (data not shown), up to 200 μM H_2O_2, was chosen for further experiments.

Results of the analysis of the effect of H_2O_2-exposure on mitochondrial respiratory capacities are shown in Fig. 6. Incubation of H9C2 cells with H_2O_2 resulted in a significant dose-dependent decrease of endogenous DNP-uncoupled respiration rate in intact cells (Fig. 6A), which was reduced by 17%, 45% and 59%, at concentrations of 50, 100 and 200 μM H_2O_2, respectively. Consistently, H_2O_2-exposure caused a marked inhibition of maximal respiratory fluxes by NAD-dependent substrates (decreased by 48% and 59% at 100 and 200 μM H_2O_2, respectively) (Fig. 6B) and by succinate (decreased by 36% and 33% at 100 and 200 μM H_2O_2, respectively) (Fig. 6C) as measured in digitonin-permeabilized cells. Pre-treatment of cells with 10 μM carvedilol resulted in a significant protective effects on H_2O_2-induced injury of respiratory chain function, since the respiratory rates were kept very close to control values even at the highest H_2O_2 concentrations. It is noteworthy that the addition of soluble cytochrome *c* (2 μM) to H_2O_2-exposed permeabilized cells leads to a partial recovery of the succinate-dependent respiration rate without having any effect on the respiratory flux by NAD-dependent substrates. Interestingly, the above protective effect on mitochondrial function was not observed when control cells

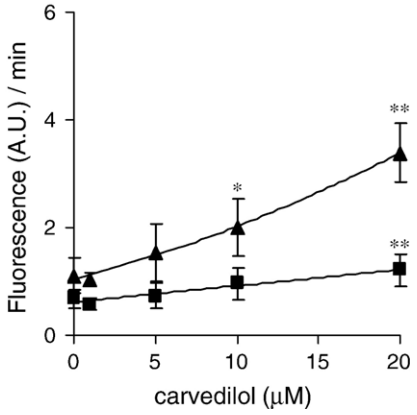

Fig. 4. Effect of carvedilol on ROS generation in intact cells. H9C2 cells were treated with carvedilol at the indicated concentrations for 24 h before measurements. ROS production rate was measured in intact cells by following the linear fluorescence increase caused by the ROS-dependent oxidation of dichlorofluorescin (DCFH) to the fluorescent compound dichlorofluorescein (DCF) either in basal condition (■) and in the presence of permeable NAD-dependent respiratory substrates (▲). The data, normalized to cell number, represent the means±SD from six independent experiments. For experimental conditions, see under Materials and methods. *$p<0.03$, **$p<0.01$ carvedilol-treated cells versus control cells.

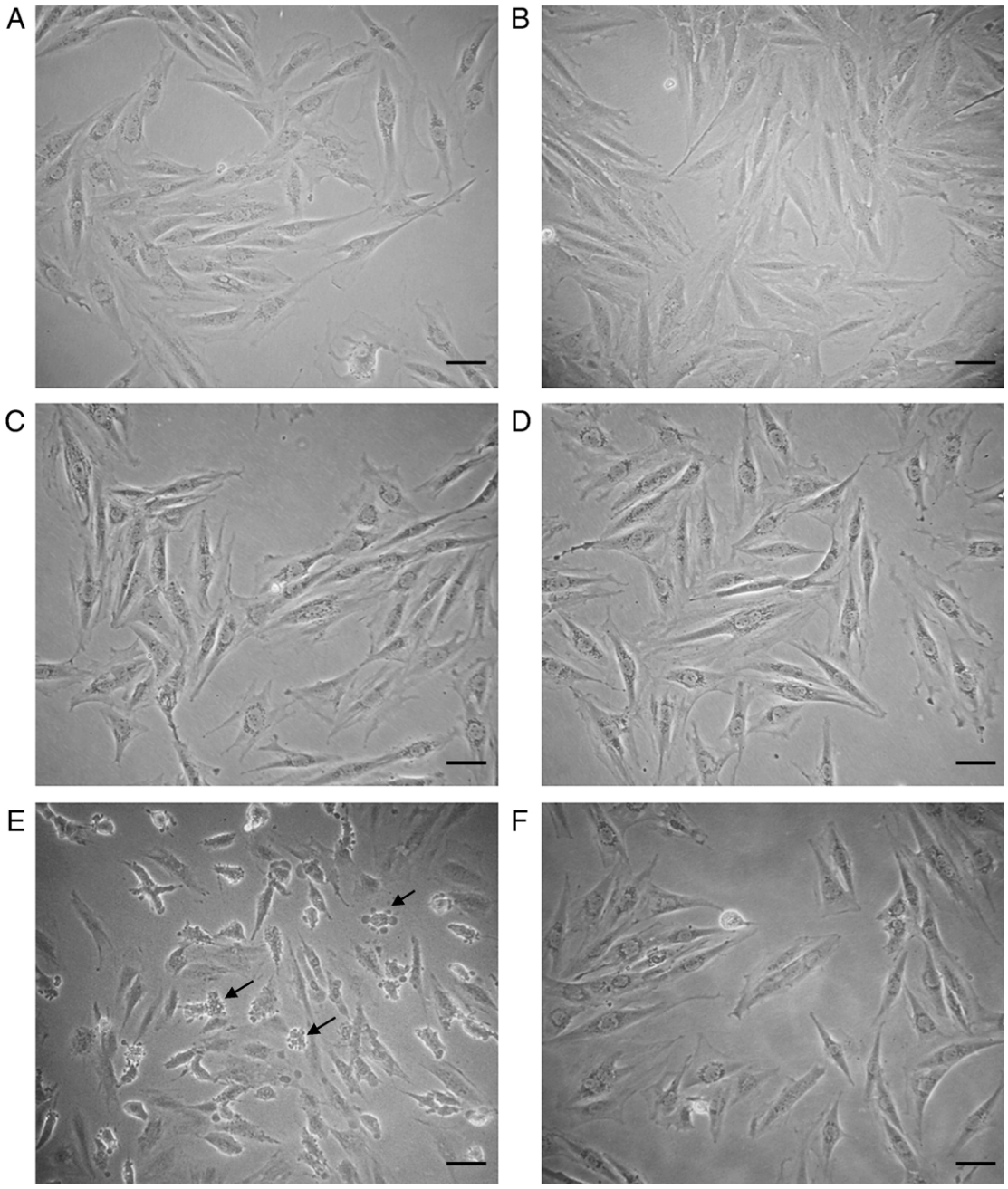

Fig. 5. Protective effect of carvedilol on morphological changes induced by H_2O_2 in H9C2 cells. H9C2 cells were pre-treated with 10 μM carvedilol for 24 h and then exposed to H_2O_2 as described in Materials and methods and examined by contrast-phase microscopy (magnification ×100). Control cells (A); carvedilol-treated cells (B); control cells, 2 h after exposure to 200 μM H_2O_2 (C); carvedilol-pretreated cells, 2 h after exposure to 200 μM H_2O_2 (D); control cells, 30 min after exposure to 500 μM H_2O_2 (E); carvedilol-pretreated cells, 30 min after exposure to 500 μM H_2O_2 (F). Each picture shows a representative field of the corresponding group. Bars: 40 μm. Small arrows, in panel E, indicate extensive cell surface blebbing.

were simultaneously incubated with 200 μM H_2O_2 and carvedilol (data not shown). This, although highlighting an indirect mitochondria-mediated protection by carvedilol, does not exclude the possibility of a synergic effect due to a direct antioxidant (chemical) action of residual membrane-embedded drug molecules in the case of pre-treated cells.

In the light of the mitochondrial complex I-specific inhibitory action of carvedilol we tried to mimic the protective effect of the drug on the H_2O_2-mediated oxidative insult by pre-treating H9C2 cells with rotenone, a widely used and well-characterized complex I inhibitor. First, we have titrated the dose-dependent decrease of the cellular respiratory fluxes by rotenone in order to select a concentration of the drug resulting in a degree of inhibition comparable to the one observed in 10 μM carvedilol-treated cells. As shown in Fig. 7, the maximal respiratory fluxes by NAD-dependent substrates in cells incubated for 24 h with 0.1 nM rotenone were decreased by 30% ($n=4$; $p=0.05$) as compared with control cells, while no significant difference could be detected when using succinate as respiratory substrate. The 0.1 nM rotenone-pretreated cells also

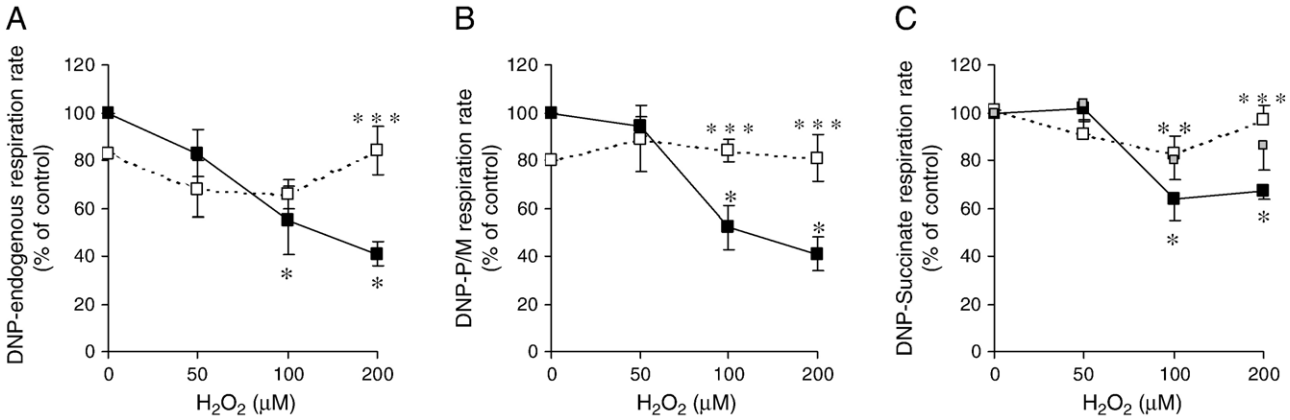

Fig. 6. Protective effect of carvedilol on H_2O_2-induced decay of mitochondrial respiration. H9C2 control cells (■) and carvedilol-pretreated (10 μM carvedilol for 24 h) H9C2 cells (□) were exposed to H_2O_2 for 2 h as described under Materials and methods. DNP-uncoupled respiration rates were measured polarographically in intact cells (A) or in digitonin-permeabilized cells in the presence of pyruvate/malate (B) or succinate (C) as respiratory substrates. Grey squares, in panel C, indicate the effect of cytochrome *c* addition on uncoupled succinate-dependent respiration rate of digitonin-permeabilized cells. The data represent the means±SD from five independent experiments. For experimental conditions, see under Materials and methods. *$p<0.001$, H_2O_2-exposed cells versus control cells; **$p<0.05$, ***$p<0.001$, carvedilol-pretreated H_2O_2-exposed cells versus H_2O_2-exposed cells.

showed an increase of ROS production rate as compared with control cells (data not shown). The rotenone-pretreated cells were protected from the H_2O_2-induced decay of mitochondrial respiratory fluxes (Fig. 7).

The effect of carvedilol pretreatment on intracellular total glutathione (GSH), oxidized fraction of glutathione (GSSG) and protein sulfhydryl groups (PSH) content in H_2O_2-exposed cells was also evaluated and the results are reported in Fig. 8. Incubation of H9C2 cells with H_2O_2 resulted in a dramatic dose-dependent decline of GSH and of PSH content which decreased

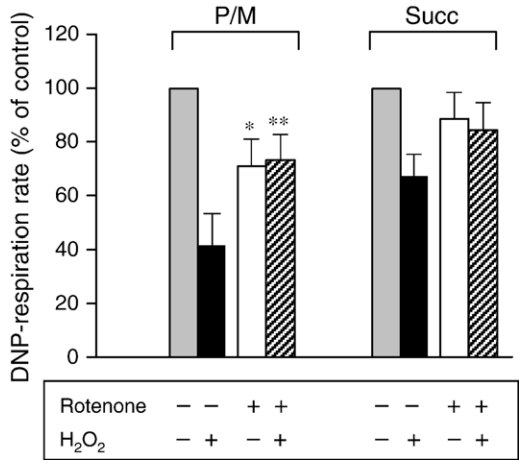

Fig. 7. Protective effect of partial inhibition of complex I by a sub-saturating rotenone concentration on H_2O_2-induced decay of mitochondrial respiration. H9C2 cells were pre-treated with 0.1 nM rotenone for 24 h and then exposed to 200 μM H_2O_2 as described in Materials and methods. DNP-uncoupled respiration rates by pyruvate/malate or succinate were measured polarographically in control cells (grey bars), cells exposed to H_2O_2 (filled bars), rotenone-treated cells (open bars) and rotenone-pretreated cells exposed to H_2O_2 (hatched bars) as described under Materials and methods. Mean values from four independent experiments±SD, are expressed as percentage of control cells. *$p<0.05$, rotenone-treated cells versus control cells; **$p<0.02$, rotenone-pretreated H_2O_2-exposed cells versus H_2O_2-exposed cells.

by 37%, 54% and 70% and by 29%, 50% and 67% at 50, 100 and 200 μM H_2O_2, respectively, as compared with control cells. In parallel a four-, six- and seven-fold increase of GSSG cellular content with 50, 100 and 200 μM H_2O_2, respectively, was observed. Pretreatment of cells with carvedilol resulted in a partial but significant recovery of GSH and PSH content and in a reduction of GSSG amount as compared to H_2O_2-exposed cells. In particular, in carvedilol pretreated cells, the cellular contents of GSH and PSH were both increased by as much as 46% at 200 μM H_2O_2, as compared with H_2O_2-exposed cells. A relative decrease by up to 68% of GSSG content was observed at the same H_2O_2 concentration in carvedilol pretreated cells as compared with untreated H_2O_2-exposed cells.

We have also measured GSH, GSSG and PSH content in rotenone-pretreated and in rotenone-pretreated, H_2O_2-exposed cardiomyocytes. No significant difference was detected between control cells and rotenone-pretreated cells before or after H_2O_2-exposure, with the exception of a significative increase (35%, $p=0.021$) of PSH content in rotenone-pretreated, H_2O_2-exposed cells vs. H_2O_2-exposed control cells.

4. Discussion

In the present work we have characterized, for the first time, the impact of carvedilol-treatment on the mitochondrial functions and ROS metabolism of heart-derived H9C2 cells and its protective effect against H_2O_2-induced oxidative stress. It has to be pointed up that, although the effect of carvedilol treatment on the respiratory chain activity has already been described in isolated mitochondria [24,25,32], the increasing evidences of the crucial importance of the functional interactions of these organelles with cytoskeleton, other subcellular organelles and cytosol motivate the need to evaluate the effect of the drug under conditions approximating more closely the in vivo situation. For this reason, we have standardized, for the first time, the methods for a detailed analysis of mitochondrial

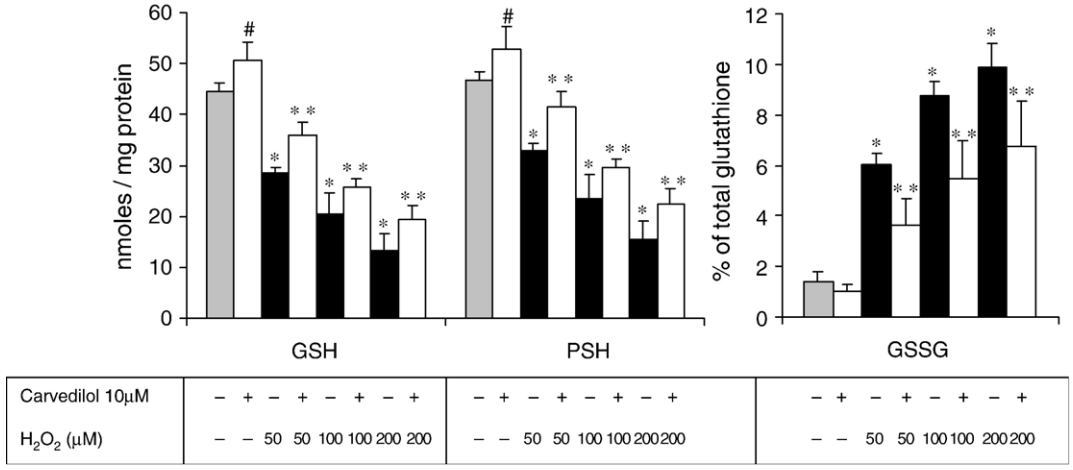

Fig. 8. Effect of carvedilol on total glutathione (GSH), oxidized glutathione (GSSG) and protein sulfhydryl groups (PSH) content in H_2O_2-induced oxidative stress. The experimental conditions are the same described in the legend to Fig. 6. Total glutathione (GSH), protein thiol groups (PSH) and oxidized glutathione (GSSG) contents were measured in total cell lysates of control cells (grey bars) and of cells exposed for 2 h to H_2O_2 without (filled bars) or after (open bars) 24 h pre-treatment with 10 μM carvedilol. The data represent the means±SD from five independent experiments. For experimental conditions, see under Materials and methods. $^{\#}p<0.05$, carvedilol-treated cells versus control cells; $^{*}p<0.001$, H_2O_2-exposed cells versus control cells; $^{**}p<0.01$, carvedilol-pretreated H_2O_2-exposed cells versus H_2O_2-exposed cells.

respiratory capacities and OXPHOS efficiency in intact and digitonin-permeabilized H9C2 cells (Fig. 1), thus keeping the organelles in a more physiological environment. The concentrations of carvedilol used in our experiments and already reported by other groups [24,32–35], were chosen considering that although a carvedilol plasma concentration of 0.3 μmol/L was reached after one oral dose of 50 mg [36], the concentration in cellular membranes may be much higher on the basis of its lipophilic structure [37].

In our experimental conditions we have found a significant dose-dependent decline of mitochondrial respiratory fluxes by carvedilol-treatment. The decrease of the respiration rate by endogenous substrates in intact cells was more pronounced when measured in the presence of the uncoupler DNP, i.e. under maximal respiratory fluxes, as compared with basal respiration rates. The data also indicate a dose-dependent decrease of the stimulatory effect of the uncoupler DNP on the endogenous respiration rates. This could be explained by a partial uncoupling effect of carvedilol, in agreement with previous results obtained with isolated mitochondria [24,32]. This was also confirmed by a slight (− 20%) but significant ($p<0.03$) decrease of the RCR values by NAD-dependent substrates in carvedilol-treated cells as compared with control cells. The maximal oxygen consumption rates (in the presence of DNP) in digitonin-permeabilized cells, exhibited a significant decrease only in the presence of NAD-dependent respiratory substrates. These results would suggest a specific inhibition by carvedilol on the upstream segment of the mitochondrial respiratory chain. In fact, by measuring the specific enzymatic capacities of the mitochondrial respiratory complexes, we have detected a consistent decline of complex I activity subsequent to carvedilol-treatment. The previously reported inhibitory effect of carvedilol on isolated mitochondria as well as on purified complex I [24], would favour the hypothesis of a direct off-target action of the drug on the activity of this respiratory complex by a still unclear mechanism. On the other hand, the lack of effect on mitochondrial bioenergetic function in animal models [38,39] could be ascribed to the treatment procedure and possibly to the different concentrations reached by the drug in the cardiac tissue.

The respiratory complex I and complex III are the major sites for the production of oxygen radical species and their defective activities could cause a significant increase in ROS production [21,40]. Indeed, an increase of oxygen radicals production associated with the carvedilol-inhibition of the steady-state respiration by NAD-dependent substrates has been previously shown in isolated mitochondria [24]. The increase of cellular ROS production observed in carvedilol-treated cells respiring on endogenous substrates (Fig. 4) could be possibly associated with the above inhibition of the mitochondrial complex I. This effect becomes, in fact, more evident in the presence of saturating concentrations of cell permeant NAD-dependent substrates (Fig. 4) which leads to a higher electron pressure on complex I and to an increase in the reduction level of its redox centers. It is worth noting that the observed increase in ROS production could be also due to a lowered efficiency of the scavenging apparatus. However, no variations were found in the catalase and Cu/Zn superoxide dismutase activities (data not shown). Actually, an increase of PSH and of total glutathione (GSH) was detected in carvedilol-treated cells without any influence on the GSSG relative amount. This finding is in line and extends previous observation by Oliveira et al. who have demonstrated, in isolated mitochondria, a specific protective effect of carvedilol against the oxidation of mitochondrial membrane PSH resulting in the final inhibition of the MPT pore opening [23].

Some of the carvedilol effects have been assigned to its antioxidant properties [6,12] which might contribute to the cardio-protective action. The antioxidant activity of carvedilol has been tested by studying the effects of carvedilol (10 μM)

pre-treatment on the oxidative insult by exogenous hydrogen peroxide, one of the major reactive oxygen intermediates produced in the failing myocardium [41]. The exposure of control cells to H_2O_2 induces a dose-dependent inhibition of the maximal respiratory capacities by endogenous and exogenous substrates (either pyruvate+malate or succinate) as measured in intact and in digitonin-permeabilized cells, respectively (Fig. 6). Strikingly, pre-treatment of H9C2 cells with carvedilol results in a marked protection against H_2O_2-mediated cytotoxicity (Fig. 6). The lack of protection in cells simultaneously exposed to carvedilol+H_2O_2 excludes the possibility of an effect solely due to a direct antioxidant action of the drug.

A digitonin-induced cytochrome *c* release from mitochondria [42] could be also involved in the lower mitochondrial respiration rates observed in permeabilized H_2O_2-exposed cells. Indeed, the addition of cytochrome *c*, while having no effect on control cells and on H_2O_2-exposed cells respiring on NAD-dependent substrates, substantially recovered the H_2O_2-dependent decline of succinate-elicited respiration rate (Fig. 6C). The absence of a stimulatory effect of exogenous cytochrome *c* on pyruvate/malate-dependent respiration could indicate a full control by complex I on the respiratory flux, under these conditions of mitochondrial damage, which would override any effect of a partial cytochrome *c* depletion. On the other hand, the possibility that the loss of cytochrome *c* could specifically occur in the unphysiological condition of a rotenone-inhibited respiratory chain utilizing succinate as electron donor, cannot be excluded [43,44].

Incubation of H9C2 cells with H_2O_2 results also in a dose-dependent decline of total glutathione (GSH) and PSH content with a parallel increase of GSSG cellular content (Fig. 8). Pre-treatment of cells with carvedilol induces a partial, but significant maintenance of total glutathione (GSH) and PSH cellular content and in a decrease of GSSG as compared to H_2O_2-exposed cells. This effect could be of the utmost importance in conditions where the prevention of protein thiol oxidation by carvedilol would protect from the apoptotic cell death caused by the calcium-induced mitochondrial permeability transition pore (MPTP) [23,25]. It is also important to underline that complex I is known to possess critical active thiol groups which make this complex particularly vulnerable to oxidative modification [45]. The observed recovery of maximal respiratory fluxes in carvedilol pretreated H_2O_2-exposed cells, respiring on endogenous and exogenous substrates, could be directly related to the decrease of the oxidized glutathione fraction (GSSG) (Fig. 8). The ability of carvedilol to preserve the redox status of critical mitochondrial proteins has also been proposed to explain its protective effect against doxorubicin-induced mitochondrial cardiotoxicity [35,38,39]. In this condition, cardiac mitochondria are implicated as primary targets of doxorubicin toxicity, which is likely to be mediated by highly reactive oxygen free radical species generated from mitochondrial complex I.

The mild pro-oxidant effects of carvedilol and its beneficial H_2O_2-protective activity could appear somehow contradictory. However, a preconditioning-like mechanism could well conciliate this Janus-faced action of carvedilol.

The "ischemic preconditioning" was initially described by Murray and colleagues in 1986 [46], but the concept has thereafter been extended also to non-ischemic stress conditions such as hypoxia, stretch and exposure to reactive oxygen radicals and chemicals [31,47–49]. This mechanism now represents a well-established tool for studying oxidant pathways of protection and injury [50]. Recently, a moderate increase in ROS promoted by antiapoptotic Bcl-2 family members has been suggested to be one of the mechanisms to prevent cell death, by enhancing antioxidant defences and preparing cells to a better response against acute oxidative stress [51].

Therefore, the carvedilol-dependent inhibition of complex I and the associated production of sublethal levels of ROS could produce preconditioning cytoprotection against subsequent oxidative damage possibly by modulating the antioxidant response and/or the redox state of specific protein or lipid messengers. The similar protective effect from H_2O_2-mediated oxidative insult observed in H9C2 cells pre-treated with a sub-saturating concentration of rotenone (Fig. 7), however, is not associated with any change of GSH, GSSG and PSH content in rotenone-pretreated cells. This would suggest that the pre-conditioning effect could occur by a different mechanism possibly involving enzymatic ROS-scavenging activities not analyzed in the present paper. The significative increase of PSH content (+35%, $p=0.021$) in rotenone-pretreated, H_2O_2-exposed cells vs. H_2O_2-exposed control cells might put in evidence an important role of protein sulfhydryl groups in the antioxidant effect.

In conclusion, on the basis of our results, the protection of rat heart H9C2 cells from H_2O_2-induced damage can be ascribed to a double action of carvedilol, i.e. a direct antioxidant effect and a preconditioning-like mechanism via mitochondrial complex I inhibition. Induction of key cellular antioxidants by carvedilol in cardiovascular cells might be an important mechanism underlying its protective effects observed in various cardiovascular disorders.

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