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Carvedilol Inhibits the Exogenous NADH Dehydrogenase in Rat Heart Mitochondria

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There are several reports on the oxidation of external NADH by an exogenous NADH dehydrogenase in the outer leaflet of the inner membrane of rat heart mitochondria. Until now, however, little was known about its physiological role in cellular metabolism. The present work shows that carvedilol ({1-[carbazolyl-(4)-oxy]-3-[2-methoxyphenoxyethyl)amino]-propanol-(2)}) is a specific inhibitor of an exogenous NADH dehydrogenase in rat heart mitochondria. Carvedilol does not affect oxygen consumption linked to the oxidation of succinate and internal NADH. It is also demonstrated that the inhibition of exogenous NADH dehydrogenase by carvedilol is accompanied by the inhibition of alkalinization of the external medium. In contrast to the addition of glutamate/malate or succinate, exogenous NADH does not generate a membrane potential in rat heart mitochondria, as observed with a TPP^+ electrode. It is also demonstrated that the oxygen consumption linked to NADH oxidation is not due to permeabilized mitochondria, but to actual oxidase activity in the inner membrane. The enzyme has a K_m for NADH of 13 μM. Carvedilol is a noncompetitive inhibitor of this external NADH dehydrogenase with a K_i of 15 μM. Carvedilol is the first inhibitor described to this organospecific enzyme. Since this enzyme was demonstrated to play a key role in the cardiotoxicity of anticancer drugs of the anthracycline family (e.g., adriamycin), we may suggest that the administration of carvedilol to tumor patients treated with adriamycin might be of great help in the prevention of the cardioselective toxicity of this antibiotic. © 2000 Academic Press

Key Words: **rat heart mitochondria; exogenous NADH dehydrogenase; inhibitor; carvedilol.**

In contrast to yeast or plants, where the presence of exogenous NADH dehydrogenases is not a matter of discussion, the existence of an enzyme of that kind in animals, namely in heart, is a source of debate and few studies have been made so far regarding the possible physiological role of the enzyme or its action in different pathological processes.

It is a well known fact that NAD^+ and NADH do not permeate the inner mitochondrial membrane (as described by Lehninger (6)), but several reports show an oxygen consumption associated with the oxidation of external NADH in yeast and plants. In the first case, the existence of an exogenous NADH dehydrogenase in the cytoplasmic leaflet of the inner membrane has been demonstrated. This enzyme is responsible for the oxidation of exogenous NADH, delivering electrons to the respiratory chain through the ubiquinone pool. In plants, the presence of external NADH dehydrogenases, sometimes coupled with nonphosphorylating pathways, was confirmed and their role has been studied with some detail for the past several years (2–5, 7). With animals, however, very little is known about specific NADH dehydrogenases that directly promote oxidation of exogenous NADH in the inner mitochondrial membrane. The existence of this enzyme has been studied in the cardiac muscle; published works by Rasmussen (8, 9) and by Nohl (10, 11) present similar conclusions. The presence of an external NADH dehydrogenase in the outer leaflet of the inner mitochondrial membrane is responsible for a high respiratory rate linked to the oxidation of external NADH, albeit not coupled with ADP phosphorylation (9, 11). This high respiratory rate was shown to be inhibited by typical respiratory chain inhibitors (8–11). The inhibition by cyanide proved that electrons from external NADH are delivered to oxygen in cytocrome oxidase. It was also pointed out by Nohl and Schonheit (11) that the oxidation of exogenous NADH is associated with

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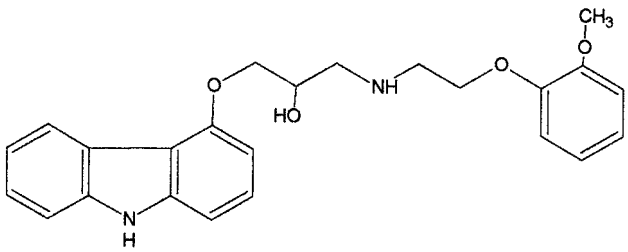

FIG. 1. Structure of carvedilol ({1-[carbazolyl-(4)-oxy]-3-[2-methoxyphenoxyethyl)amino]-propanol-(2)}).

the disappearance of protons from the medium, being those protons used by cytochrome oxidase to produce water.

As far as its physiological role, Nohl and Schonheit (11) propose that this external NADH dehydrogenase might be responsible for the oxidation of external NADH during its excessive accumulation in the cytosol. Alternatively, this enzyme has been associated with some pathological conditions related to the production of oxygen free radicals released during normal metabolic events (12), under conditions of ischemia/reperfusion (13), and most of all with the cardioselective toxicity of adriamycin, an anticancer drug that accepts the reducing equivalents from the external NADH dehydrogenase (14).

A specific inhibitor of the internal NADH dehydrogenase, namely ethoxyformic anhydride (EFA),[2] has been used to descriminate between the internal and external oxidase activities (10–12), although it was shown previously that EFA also inhibits Complex III (15). The discovery of a specific inhibitor for this exogenous dehydrogenase could be a key to identifying this enzyme in rat heart mitochondria. The aim of this work was to study the effect of carvedilol ({1-[carbazolyl-(4)-oxy]-3-[2-methoxyphenoxyethyl)amino]-propanol-(2)}) (Fig. 1), a cardioprotective drug also shown to protect mitochondrial bioenergetics during oxidative stress (16), on the activity of the external rat heart NADH dehydrogenase, in an attempt to demonstrate its selective inhibitory actions.

MATERIALS AND METHODS

Carvedilol was obtained from Boehringer (Mannheim, Germany) and potassium cyanide from Merck (Darmstadt, Germany); all other coumponds were purchased from Sigma Chemical Co. (St. Louis, MO). Carvedilol was prepared in dimethylsulfoxide (DMSO). Rat heart mitochondria from male Wistar rats (250–300 g) were prepared using a conventional procedure (20), with slight modifications. Briefly, the rats were sacrificed by cervical dislocation and the hearts were imediately excised and minced finely in an ice-cold isolation medium containing 250 mM sucrose, 1 mM EGTA, 10 mM Hepes–KOH (pH 7.4), and 0.1% defatted BSA. The minced blood-free tissue was then ressuspended in 40 ml of isolation medium containing 1 mg protease Type VIII (Sigma No. P-5390) per mg of tissue and homogenized with a tightly fitted homogenizer (Teflon:glass pestle). The suspension was incubated for 1 min (4°C) and then rehomogenized. The homogenate was then centrifuged at 10,000*g* for 10 min (Sorvall RC-5C, Plus, SS 34 rotor, 4°C). The supernatant fluid was decanted and the pellet, essentially devoid of protease, was gently homogeneized to its original volume with a loose-fitting homogeneizer. The suspension was centrifuged at 500*g* for 10 min and the resulting supernatant was centrifuged at 10,000*g* for 10 min. The pellet was resuspended using a paint brush and repelleted twice at 10,000*g* for 10 min. EGTA and defatted BSA were omitted from the final washing medium. Mitochondrial protein content was determined by the biuret method calibrated with BSA. Oxygen consumption of isolated mitochondria was monitored polarographically with a Clark oxygen electrode connected to a suitable recorder in a 1-ml thermostated water-jacketed closed chamber with magnetic stirring at 25°C. The standard respiratory medium consisted of 300 mM sucrose, 2 mM EDTA, 20 mM triethanolamine, and 2.1 mM KH_2PO_4, pH 7.4. Mitochondria were suspended at a concentration of approximately 1 mg/ml in the respiratory medium. State IV respiration was measured in the presence of 10 mM glutamate/malate, 8 mM succinate (plus 2 μM rotenone), or 5 mM NADH. State III respiration was initiated by adding ADP in excess (1 mM). The respiratory control ratio (RCR) was calculated using oxygen consumption rates during state III and subsequent state IV respiration. The mitochondrial transmembrane potential ($\Delta\Psi$) was estimated with a TPP^+ electrode acording to Kamo *et al.* (21) without correction for the "passive" binding contribution of TPP^+ to the mitochondrial membranes because the purpose of the experiments was to show relative changes in the potential rather than absolute values. A matrix volume of 1.1 μl/mg protein was assumed. Reactions were carried out at 25°C in 1 ml of the standard respiratory medium supplemented with 3 μM TPP^+ and 1 mg of mitochondria. Respiratory rates and $\Delta\Psi$ were measured simultaneously. The effect of carvedilol was studied with a preincubation of 3 min at 25°C. Proton movements were followed with mitochondria (1 mg) suspended in 1 ml of a reaction medium consisting of 150 mM sucrose, 75 mM KCl, 2 mM glycylglycine, 1 mM EGTA, pH 7.4 (at 25°C), using a Jenway Model 3305 pH meter connected to a suitable recorder. Valinomycin (3 μg/mg protein) was added to eliminate any transmembrane electric potential. An oxygen electrode was inserted in the reaction cell to follow pH changes and the respiratory rates, simultaneously. The effect of carvedilol was studied after a preincubation period of 3 min. When using glutamate/malate or NADH as substrates, total deenergization of mitochondria was achieved before each assay. Mitochondrial integrity was determined by the citrate synthase assay, as described previously (17, 18). K_m and K_i were determined using the initial rate of oxygen consumption versus the substrate (NADH) concentration in the presence or absence of the inhibitor (50 μM), according to Lineweaver–Burk plots.

RESULTS

As can be seen in Fig. 2, the addition of external NADH to RHM caused an increase in oxygen consumption which is greater than when succinate or glutamate/malate were used, consistent with that previously described by Nohl (11). Some classic inhibitors, such as rotenone, antimycin A, cyanide, azide, or myxothiazol caused a total inhibition on the respiratory rate. After azide addition a slight oxygen consumption

[2] Abbreviations used: BSA, bovine serum albumine; RCR, respiratory control ratio, TPP^+, tetraphenylphosphonium cation, K_m, Michaelis–Menten constant, K_i, inhibition constant; RHM, rat heart mitochondria; DNP, 2,4-*p*-dinitrophenol; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; DOC, sodium deoxycolate; EFA, ethoxyformic anhydride.

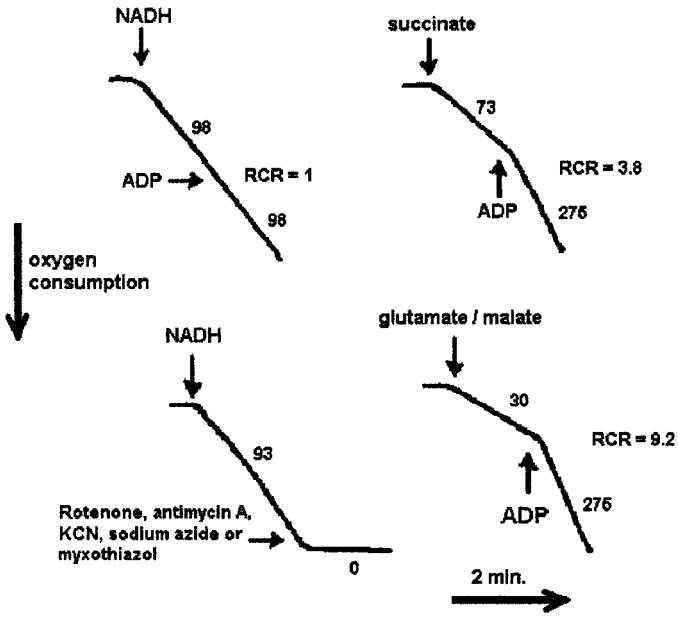

FIG. 2. Typical traces of oxygen consumption stimulated by external NADH, glutamate/malate, and succinate. State IV was initiated with 5 mM NADH, 10 mM glutamate/malate, or 8 mM succinate. ADP in excess (1 mM) was added to stimulate state III respiration. The effect of several inhibitors on the oxygen consumption sustained by exogenous NADH is also shown (0.5 μg antimycin A, 1 mM KCN, 2 μM rotenone, 200 μM sodium azide, or 10 μM myxothiazol). The numbers adjacent to the curves indicate *n* atoms oxygen/minute/mg protein.

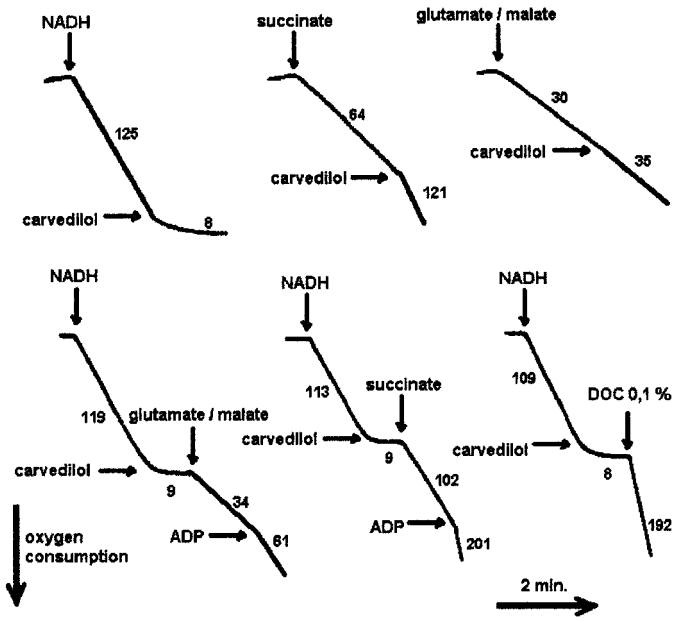

FIG. 4. Typical traces of the effect of carvedilol (100 μM) on oxygen consumption sustained by external NADH, glutamate/malate, and succinate in RHM (1 mg/ml). Substrate concentrations were as indicated in Fig. 2. Also shown is the effect of adding the detergent DOC (0.1% final concentration) on the respiratory rate sustained by external NADH (5 mM) inhibited by 100 μM carvedilol. The numbers adjacent the curves indicate *n* atoms oxygen/minute/mg protein.

could still be observed, which is in agreement with Slater (19). ADP gave rise to the transition to state III respiration, with a RCR of 1 for NADH (no coupling), and about 3 for succinate and 7 for glutamate/malate (Fig. 3). The RCR values for glutamate/malate and succinate were indicative of a good mitochondrial preparation (20), which was also confirmed by the citrate synthase assay, where about 4% of damaged mitochondria were found (data from three independent preparations). The addition of NADH to the medium in the absence of mitochondria did not stimulate oxygen consumption (data not shown). As previously described by Nohl and Schonheit (11), uncouplers like DNP have no effect in the respiratory rate induced by external NADH. In our own experiments, the addition of 0.5 μM FCCP decreased the oxygen consumption by 5–10% (data not shown). Increasing concentrations of calcium did not cause any alteration in state IV respiration sustained by NADH.

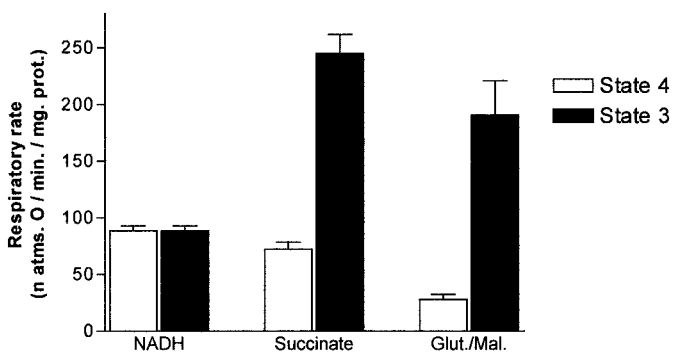

FIG. 3. Comparison between state IV and state III respiration for external NADH, succinate and glutamate/malate in the conditions of Fig. 2. Data are mean ± SE for three independent experiments.

The addition of 100 μM carvedilol to RHM consuming external NADH caused a near total inhibition of the oxygen consumption, as can be seen in Fig. 4. The slight oxygen consumption observed after the addition of carvedilol was due to the oxidation of endogenous substrates, which is not affected by the drug. A similar carvedilol concentration caused a stimulation by almost 60% of state IV respiration induced by succinate and a 10% stimulation when glutamate/malate was used (Figs. 4 and 5). However, it is apparent in Fig. 4 that after the inhibition of NADH supported respiration by carvedilol, the addition of succinate or glutamate/malate restored oxygen consumption. ADP increased the respiratory rate, though with a lower RCR. To separate internal and external sites of NADH consumption, we permeabilized RHM with DOC 0.1% after inhibition of the exogenous NADH dehydrogenase with 100 μM carvedilol. The result was a sudden in-

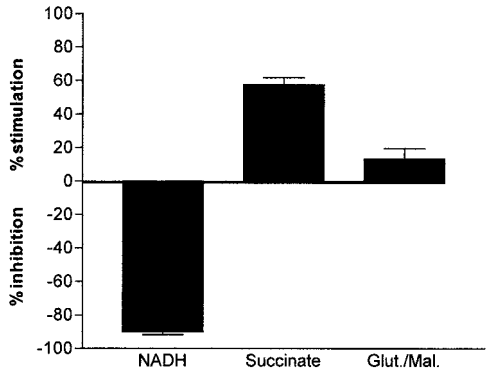

FIG. 5. Effect of 100 μM carvedilol on the oxygen consumption sustained by external NADH, glutamate/malate, and succinate in RHM (1 mg/ml). Substrate concentrations were as described in Fig. 2. The effect (inhibition or stimulation) was calculated as the difference in respiratory rate before and after the addition of carvedilol. Data are mean ± SE of three independent experiments.

crease in oxygen consumption due to the oxidation of NADH by the internal NADH dehydrogenase.

Rat liver mitochondria displayed very small oxygen consumption values linked to the oxidation of external NADH which were not inhibited by carvedilol (data not shown).

The carvedilol inhibition of cardiac mitochondrial respiratory rate sustained by 5 mM external NADH is concentration dependent (Fig. 6). In this case, instead of being applied after the addition of NADH, we preincubated mitochondria with the drug for 3 min.

Figure 7 shows the simultaneous measurement of cardiac mitochondrial membrane potential and oxygen consumption. It is noteworthy that external NADH did not generate any membrane potential (no TPP^+ uptake). The small increase in the register was due to an addition artifact, as confirmed by the same effect in the absence of mitochondria (not shown). However, the addition of glutamate–malate developed a membrane potential of about −210 mV (without correction for passive membrane binding). ADP addition caused an increase in the respiratory rate and an expected drop in the membrane potential, related to its phosphorylation. The same figure shows that mitochondria also developed a membrane potential in response to glutamate–malate after adding carvedilol, although to a lesser extent (see Discussion). Carvedilol inhibited mitochondria were also capable of phosphorylating added ADP, but again, in a suspressed manner.

Carvedilol also inhibited in a dose-dependent manner the media alkalinization induced by exogenous NADH oxidation (Fig. 8). The inhibition of oxygen consumption was accompanied simultaneously by an inhibition in the rate of media alkalinization. As seen in Fig. 9, we observed a larger inhibiton of the alkalinization rate of the media as compared to the inhibition of oxygen consumption.

The Michaelis–Menten constant (K_m) and the inhibiton constant (K_i) were estimated by a Lineweaver–Burk analysis based on the measurement of initial oxygen consumption for several NADH concentrations with and without added carvedilol (Fig. 10). The shape of the plot is typical of a noncompetitive inhibition. Standard equations were used in order to calculate the kinetic constants (22). K_m was calculated to be 13 μM of NADH, while the estimated K_i was 15 μM for carvedilol.

DISCUSSION

The existence of this exogenous NADH dehydrogenase in rat heart mitochondria raises important questions related to its physiological role. The enzyme oxidizes external NADH at the level of complex I, delivering electrons to cytochrome oxidase without generating a protomotive force. Acording to what is currently known it is striking that electrons can be carried from complex I to IV without the simultaneous proton pumping to generate an electrochemical potential across the inner mitochondria membrane.

It is well established that plants and yeast can oxidize external NADH. In plants, for instance, there is a rotenone-insensitive Ca^{2+}-dependent external NADH dehydrogenase (2, 7) that is not coupled to normal phosphorylating pathways (4, 7). The existence of such an enzyme in animals is, however, still controversial. In liver mitochondria, the oxidation of external NADH has been attributed to the existence of a system containing NADH–cytochrome b_5 reductase/cytochrome b_5 in the outer mitochondrial membrane or with the electron shuttle between the two mitochondrial membranes by desorbed cytochrome c (23, 24). In a recent article, Bodrova *et al.* (25) pointed out that, under some

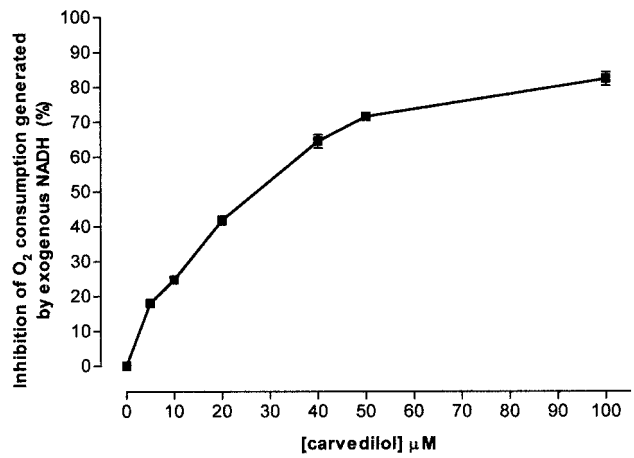

FIG. 6. Effect of different concentrations of carvedilol on oxygen consumption sustained by 5 mM external NADH. RHM (1 mg/ml) were preincubated with carvedilol for 3 min at 25°C in the reaction buffer. Data are mean ± SE of three independent experiments.

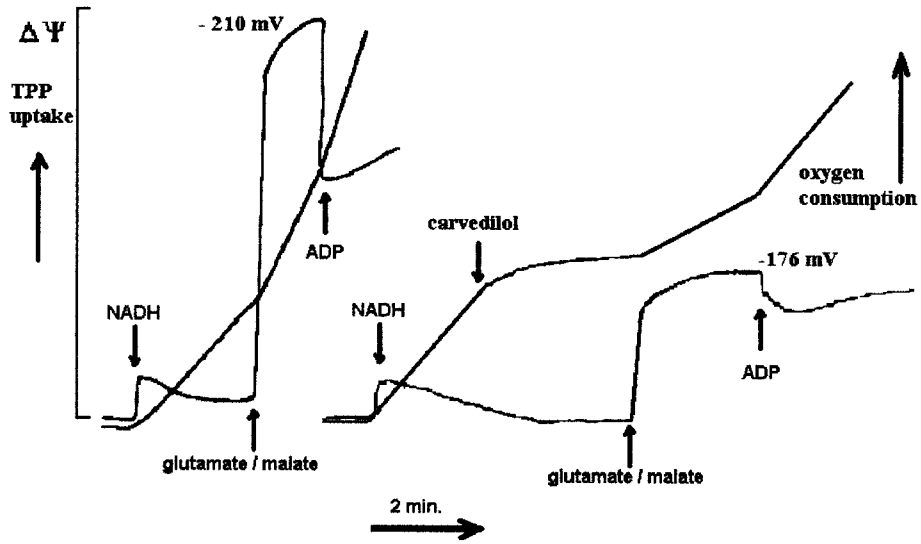

FIG. 7. Traces obtained with simultaneous measurements of mitochondrial electric potential and oxygen consumption in RHM (1 mg/ml). The concentrations of NADH and glutamate/malate were 5 and 10 mM, respectively. ADP was added in excess (1 mM). Respiration stimulated by NADH was inhibited by 100 μM carvedilol. The shown values of the electric potential were calculated as described under Materials and Methods.

experimental conditions, oxidation of external NADH could generate a membrane potential even without the participation of added cytochrome c.

In muscle mitochondria, especially in heart muscle, there are few works published regarding a possible exogenous NADH dehydrogenase. Rasmussen studied the external oxidation of NADH in pigeon heart mitochondria (9) while Nohl (10–14) used rat heart mitochondria. Both studies raise the question of whether there is a NADH dehydrogenase in the outer leaflet of the inner mitochondrial membrane of heart muscle. Perhaps this is a subunit of Complex I that delivers electrons to the ubiquinone pool. The electron pathway through the mitochondrial complexes is inhibited by several widely used inhibitors (rotenone, myxothiazol, antimycin A, azide, and cyanide) (9–11; Fig. 2). This implies that the electron route is from external NADH through complex I to complex IV, where electrons are delivered to the oxygen. That oxidation was not coupled to ADP phosphorylation and was insensitive to uncouplers. We demonstrated, in simultaneous measurements of membrane potential and oxygen con-

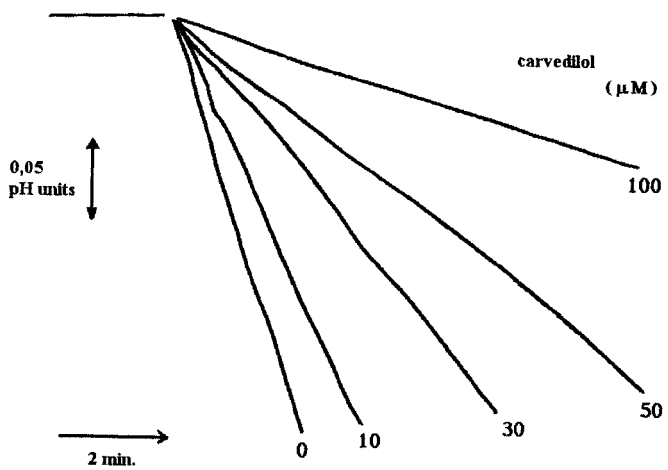

FIG. 8. Comparison of the records of pH increase in the absence and presence of increasing carvedilol concentrations. 5 mM NADH was added to the reaction media with mitochondrial protein (1 mg/ml). RHM were preincubated with carvedilol for 3 min before each assay. Valinomicin (3 μg/mg protein) was added in order to dissipate membrane potential. The record is representative of three different preparations and recorded as described under Materials and Methods.

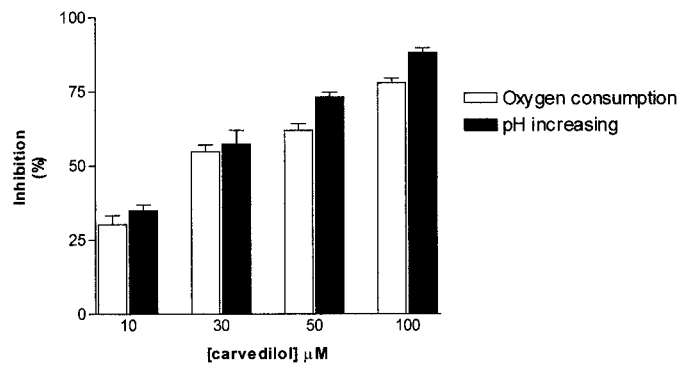

FIG. 9. Inhibitory action of carvedilol on oxygen consumption and disapperance of protons from the media. Both measurements were made at the same time under the conditions described under Materials and Methods. Data are mean ± SE of three independent experiments.

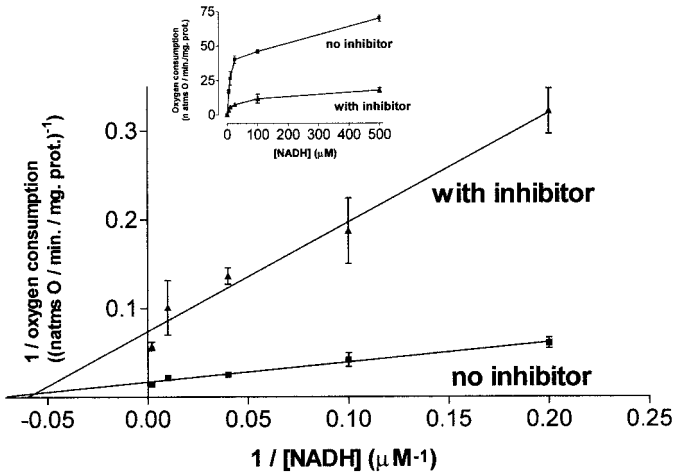

FIG. 10. Lineweaver–Burk plots of the initial rates of oxygen consumption by RHM (1 mg/ml) in the absence and presence of carvedilol (50 μM). The reactions were started with 5 mM NADH. Carvedilol was incubated with RHM for 3 min in the reaction media at 25°C. (Inset) The relation between initial oxygen consumption and [NADH] in the presence and absence of the inhibitor. The data are means of four different preparations. K_m and K_i were determined from the Lineweaver–Burk plot to be 13 and 15 μM, respectively.

sumption (Fig. 7), that there is no generation of membrane potential coupled to the oxidation of external NADH. In a study involving ischemia/reperfusion (13), Nohl *et al.* reported that the exogenous NADH dehydrogenase was mainly responsible for the generation of ROS, a fact that confirmed previous data (12), when it was discovered that the so-called exogenous NADH oxido-reductase is a novel and very active generator of superoxide anion in rat heart mitochondria. This novel enzyme was subsequently implicated to be responsible for the selective cardiotoxicity of anthracyclines like adriamycin (13, 14).

Until now, no inhibitor was known to selectively inhibit this enzyme. In this study, we demonstrate that carvedilol ({1-[carbazolyl-(4)-oxy]-3-[2-methoxyphenoxyethyl)amino]-propanol-(2)}) (Fig. 1), a cardioprotective drug that also protects mitochondrial function during the generation of free radicals (for a review see 16), inhibits the oxygen consumption linked to the oxidation of external NADH. Carvedilol does not inhibit oxygen consumption linked to internal NADH or succinate. With external NADH, near 90% inhibition of the respiratory rate was attained. The low respiratory rate after the addition of 100 μM carvedilol may be due to the oxidation of endogenous substrates, not affected by the drug. The large increase in oxygen consumption after the addition of DOC 0.1% (Fig. 4) to the inhibited exogenous NADH-sustained respiration indicates that the destruction of the inner mitochondrial membrane allows access of exogenous NADH to the internal NADH dehydrogenase of Complex I. This can be considered as a key evidence for the existence of an exogenous NADH dehydrogenase in rat heart mitochondria.

Simultaneous measurements of membrane potential and oxygen consumption confirmed the reason for the nonphosphorylation of ADP: the oxidation of exogenous NADH is not coupled to the generation of membrane potential (Fig. 7). Following carvedilol (100 μM) inhibition of the respiratory rate sustained by exogenous NADH, RHM were still capable of developing a membrane potential by oxidizing glutamate–malate, albeit at a lower potential (ca., 34 mV lower). Carvedilol decreases the potential generated by succinate or glutamate/malate (16), which is associated with the stimulation of respiratory rates sustained by those substrates. According to other results in our laboratory (manuscripts in preparation), the drop in the membrane potential is caused by an increased H^+ leak, either by a weak protonophoric action or by disruption of membrane order. Both possibilities can explain the increased proton leak in the presence of carvedilol and the drop in membrane potential accompanied by a stimulation of the respiratory rate. It is worth noting that the carvedilol concentrations used for preventing lipid peroxidation and oxidative damage to mitochondria during events of ROS generation (like in ischemia/reperfusion) are 5–10 μM, in which only a very small increase in proton leak is observed (16), being much more extensive in the range of concentrations used in this work (50–100 μM).

Rasmussen (9) and Nohl (11) described the disappearence of protons from the media coupled to the oxidation of external NADH, a fact that Nohl and Schonheit (11) considered to be a consequence of the use by cytochrome oxidase of those protons to reduce oxygen. We found that carvedilol inhibited the alkalinization of the media in a dose-dependent manner (Fig. 8). Comparing the inhibition of pH changes with oxygen consumption (Fig. 10), we observed that pH alterations have a larger inhibition than the oxygen consumption. One possible line of reasoning is that RHM may have a small quantity of endogenous substrates, whose oxidation supports respiration that is not inhibited by carvedilol. Regardless, inhibition of the respiratory rate associated with the oxidation of exogenous NADH is coincident with the inhibition of the pH variation caused by the drug.

The K_m for the exogenous NADH dehydrogenase was determined as 13 μM. K_i for carvedilol was calculated as 15 μM. The inhibition was found to be noncompetitive, according to Lineweaver–Burk plots. The inhibition mechanism is unknown, but it is possible that carvedilol, due to its great lipophilicity (26) disturbs the hydrophobic domains of the enzyme, which could include reversible combination with –SH groups buried in the lipid membrane.

The discovery of a specific inhibitor of the exogenous NADH dehydrogenase can be of great value to further studies on this enzyme. The physiological role of the enzyme is still unknown as pointed by Nohl and Schonheit (11), but may involve the liberation of highly reactive oxygen species in events of ischemia and reperfusion, in which case carvedilol may have important cardioprotective value.

This also opens new horizons regarding the potential protective role of the drug in the cardiotoxicity of adryamicin and other agents that are known to cause a mitochondrially mediated cardiomyopathy.

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