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# Inhibition of rat heart superoxidase dismutase activity by diethyldithiocarbamate and its effect on mitochondrial function

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Superoxidase dismutase (E.C. 1.15.1.1) (SOD)* is an ubiquitous enzyme that catalyzes the dismutation of superoxide anions $(O_2^-)^+$ to hydrogen peroxide and molecular oxygen [1]. SOD is thought to play an important role in protecting the cells against $O_2^-$ radical toxicity [2]. Maximal SOD activity has been observed in liver, adrenal and kidney tissues. Heart muscle contains a considerable amount of SOD [3], but little research has been done to ascertain its role in cardiac metabolism. Heikkila *et al.* [4] have reported that when diethyldithiocarbamate $(DDC)^+$, a powerful copper chelating agent, was injected intraperitoneally in mice, SOD activity in the brain, liver and blood was markedly lowered. SOD inhibition was also observed after incubation of DDC either with solutions of purified bovine SOD [5] or with mouse tissue homogenates that contained endogeneous SOD [4]. More recently, DDC has been found to potentiate oxygen toxicity and the *in vitro* effects of hemolytic agents [6] or the lethal effects of ozone and paraquat [7]. The present study was undertaken to investigate the effect of the administration *in vivo* of DDC on rat cardiac SOD activity. Since cardiac mitochondria are able to generate $O_2^-$ radicals [8], we have also studied the effects of cardiac SOD inhibition on mitochondrial function.

## *Materials and Methods*

Male Sprague–Dawley rats weighing 200–300 g were injected intraperitoneally (1.2 g/kg wt) with sodium DDC (Sigma Chemical Company, St. Louis, MO) in 0.9 per cent (w/v) NaCl solution. Control animals received the same volume of the saline vehicle (usually 0.5 ml). After the rats were killed by cervical dislocation, their hearts were rapidly removed and immersed in a Krebs-Henseleit oxygenated buffer [9] at 2–4°. In order to remove blood contamination, the hearts were perfused for 15 min by the non-recirculating Langendorff technique [10] using an oxygenated buffer containing 11 mM glucose as substrate [11]. At the end of each perfusion, the hearts were removed and homogenized in 180 mM KCl, 10 mM EDTA and 0.5 per cent (w/v) bovine serum albumin, pH 7.2, using an Ultra-Turrax homogenizer [12]. The homogenates were centrifuged at 1000 *g* for 5 min. The supernatants were filtered and recentrifuged at 10,000 *g* for 10 min. The supernatants were collected and recentrifuged at 40,000 *g* for 60 min to prepare cytosolic fractions. The above resulting crude mitochondrial pellets were resuspended in a medium containing 180 mM KCl and 0.5 per cent (w/v) bovine serum albumin, pH 7.2 (medium-1) and centrifuged for 10 min at 5000 *g*. The mitochondrial pellets were washed again in medium-1, and after centrifugation at 5000 *g* for 10 min, the final pellets were collected and resuspended in the medium-1. Mitochondrial oxygen consumption ($QO_2$, *n* atoms oxygen uptake in presence of ADP/mg mitochondrial protein/min), the respiratory control index (RCI) and the ADP/O ratio were calculated from the decrease of the $O_2$-partial pressure measured by a Clark electrode in a closed water jacketed at 25° (Gilson Instruments, France). The assay medium consisted of 3 mM substrate (glutamate, succinate, pyruvate or α-ketoglutarate), 250 mM sucrose, 0.5 mM EDTA, 3 mM $KH_2PO_4$ at pH 7.4. ADP (250 μM) was added to the incubation mixture to initiate mitochondrial respiration. Superoxide dismutase activity was measured by the method of Nishikimi *et al.* [13] which utilizes the inhibitory action of SOD on the rate of reduction of nitroblue tetrazolium $(NBT)^+$ by $O_2^-$ mediated by phenazine methosulfate $(PMS)^+$. The assay medium consisted of 19 mM sodium pyrophosphate buffer, pH 8.3, containing 33 μM of NBT and 86 μM NADH. After mixing briefly, 50 μl of mitochondrial or cytosolic sample was added, and after addition of 69 μM of PMS, the reduction rate of NBT was monitored at 550 nm for 2 min. For all the samples the inhibition of the reaction was kept close to 50 per cent. Malondialdehyde $(MDA)^+$ content was evaluated in mitochondrial and cytosolic fractions using thiobarbituric acid as reagent [14]. The proteins were measured by the method of Bradford [15] using serum albumin as a standard.

## *Results*

Table 1 reports the SOD activity measured in mitochondria and cytosol prepared from perfused rat hearts isolated 2 hr after intraperitoneal injection with DDC.

Both mitochondrial and cytosolic SOD activity were significantly depressed by DDC. Two hours after injection, cardiac SOD inhibition reached its highest value and remained constant for another 2 hr (data not shown). The amounts of cardiac MDA produced in the animals treated with DDC are reported in Table 2. The content of mitochondrial MDA was greatly increased in the treated ani-

* *Abbreviations used:* SOD, superoxide dismutase; $O_2^-$, superoxide anions; DDC, diethyldithio carbamate; NBT, nitroblue tetrazolium; PMS, phenazine methosulfate; MDA, malondialdehyde.

Table 1. Effect of intraperitoneal injection of DDC on rat heart SOD activities (units/mg protein)

| | Mitochondria | Cytosol |
|---|---|---|
| Control | 14.81 ± 0.08 | 74.51 ± 0.10 |
| Treated | 1.12 ± 0.06 | 14.00 ± 0.10 |

The hearts after their removal were perfused retrogradely by aorta with oxygenated buffer for 15 min to remove blood contamination. The hearts were then minced and homogenized as reported in Methods. Each value represents the mean ± S.E. of six separate experiments.

Table 2. Content of MDA in rat hearts after DDC treatment (nmoles/mg protein)

| | Mitochondria | Cytosol |
|---|---|---|
| Control | 0.92 ± 0.08 | 0.88 ± 0.06 |
| Treated | 2.01 ± 0.10 | 1.16 ± 0.09 |

Each result represents the mean ± S.E. of six separate experiments.

mals; on the contrary, in the cytosol the level of MDA was only slightly increased by the treatment with DDC with respect to the control animals. Figure 1 shows the values of oxidative phosphorylation measured in heart mitochondria extracted from both the controls and the treated animals. It can be seen that in cardiac mitochondria prepared from treated animals, the RCI, $QO_2$ and ADP/O values were depressed for all the substrates used. However, the pyruvate and α-ketoglutarate induced phosphorylation rate had a more marked decline with respect to the other substrates. In particular, the RCI values were reduced to 43 and 52 per cent in treated mitochondria, when pyruvate and α-ketoglutarate were used respectively as substrate.

*Discussion*

In accordance with Heikkila *et al.* [4, 16] who found that brain, liver and blood SOD was inhibited by DDC, the present research has demonstrated that rat heart SOD was also inhibited when DDC was injected intraperitoneally into rats. SOD is an important enzyme which protects the cells against oxygen toxicity [1], and we have reported that its function is particularly important for cardiac muscle [17]. DDC lowers the SOD activity by chelating $Cu^{2+}$ ions that are required for its catalytic activity [4] and in accordance with this mechanism, Heikkila *et al.* [4] and Misra [18] found that the inhibition was reversed by addition of $CuSO_4$. In our experimental conditions we observed that DDC inhibited cytosolic SOD a Cu-Zn enzyme [19], while mitochondrial SOD, the activity of which is both Cu–Zn (56 per cent) and Mn (44 per cent) dependent [20], was also strongly reduced by the drug. This effect of DDC on mitochondrial SOD is difficult to explain and we tend to believe that the drug may negatively affect Mn–SOD by some indirect mechanisms. For example, DDC is able to inhibit glutathione peroxidase activity [7] and this event could promote the accumulation of mitochondrial $H_2O_2$ which is toxic for SOD [21]. Another explanation could be that our analytical procedure which used intact mitochondria was more sensitive to intermembrane Cu–Zn SOD than to Mn–SOD contained in the matrix. As a consequence of the treatment of the rats with DDC, cardiac mitochondrial function became insufficient, when glutamate, succinate, α-ketoglutarate and pyruvate were used as substrates. In fact, the drug produced disturbances in the electron transport chain with depression of the mitochondrial oxygen uptake during state-3 respiration. The ability of these mitochondria to produce ATP was not compromised, but occurred at a slower rate. The effects of the drug on mitochondrial-induced respiration may be due to a derangement of mitochondrial membrane integrity, probably induced by the formation of endogenous $O_2^-$ radicals, which had not been completely neutralized by the inhibited SOD.

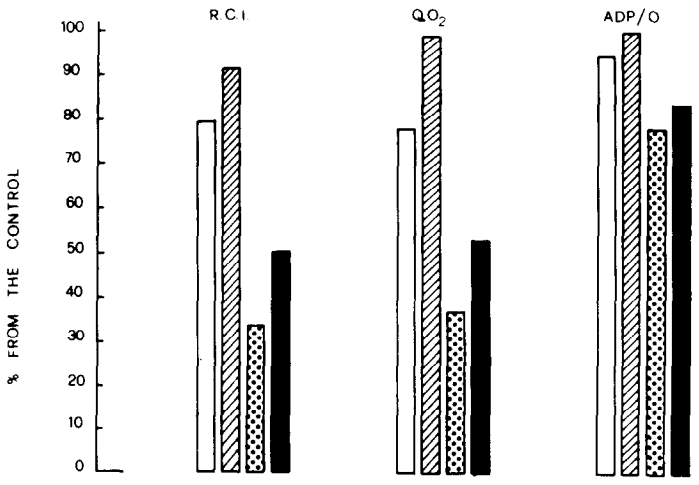


Fig. 1. Oxidative phosphorylation of cardiac mitochondria isolated from rats injected with DDC. Assay conditions are described in Methods. Control refers to mitochondria from untreated rats. Control values are: Glutamate RCI = 9.8; $QO_2$ = 160.4; ADP/O = 2.98; Succinate RCI = 3.3; $QO_2$ = 194.0; ADP/O = 1.82; Pyruvate RCI = 9.15; $QO_2$ = 129.5; ADP/O = 3.10; α-Ketoglutarate RCI = 9.8; $QO_2$ = 159.4; ADP/O = 3.10 □, Glutamate; ▨, Succinate; ▩, Pyruvate; ■, α-Ketoglutarate. Each result is mean of six separate experiments. Standard errors were less than 5 per cent of the mean values in each case.

An increase of mitochondrial MDA formation in the rats treated with DDC seems to confirm this hypothesis and suggests that polyunsaturated phospholipids may be the target of $O_2^-$ radicals toxicity. The requirement of phospholipids by membrane-bound enzymes is well documented [22] and their activities are greatly influenced by the nature of the lipid membranes [23]. Lipid peroxidation in mitochondrial membranes results in a loss of respiratory control [24] and produces lysis of mitochondria [25]. Inhibition of lipid peroxidation in isolated inner membranes of rat liver mitochondria by SOD has been described by Zimmermann *et al.* [26] and the role played by mitochondrial SOD has been confirmed by this study.

If further studies are needed to explain the mechanism of action of DDC *in vivo*, the present work supports the idea that cardiac SOD can play an important role in the preservation of heart muscle integrity, particularly at the mitochondrial level.

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