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LONIDAMINE-MEDIATED RESPIRATORY CHANGES IN RAT HEART MYOCYTES: A RE-EXAMINATION OF THE FUNCTIONAL RESPONSE OF MITOCHONDRIAL CYTOCHROME *c* OXIDASE

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Abstract—Respiratory activity of intact cardiac myocytes isolated from rats treated with lonidamine (LND) has been examined under conditions where cytochrome oxidase turns over at its maximal rate. Compared to myocytes isolated from control rat hearts, those treated with LND displayed a 60% increase in the cytochrome oxidase-dependent rate of respiration; electron microscopy revealed, in agreement with the literature, that the membrane structure of the mitochondrion had become disorganized. The increase in the rate of oxygen consumption was correlated with the (partial) impairment of the membrane ability to maintain the proton electrochemical potential gradient which normally inhibits oxidase activity. Results are discussed with reference to previous reports showing no effect of LND on cytochrome *c* oxidase activity. The evidence reported better clarifies the contribution of cytochrome oxidase to the demonstrated energetic failure displayed by cells treated with LND.

Key words: lonidamine; cytochrome oxidase; mitochondria; membrane damage

LND¶ [1-(2,4-dichlorobenzyl)-1-H-indazol-3 carboxylic acid] is a dichlorinated antispermatogenic compound [1, 2], proposed in the early 1980s as an antineoplastic drug in chemotherapy [3, 4 and references therein]. The results of experiments carried out in the past to explain the impairment of energy metabolism, always observed in tumour cells treated with LND, were basically consistent with: (1) the inhibition of the mitochondrial-bound hexokinase; (2) the inhibition of the NAD–FAD dehydrogenase complex; and (3) the structural and functional damage of mitochondrial and plasma membranes [5–7]. The metabolic impairment has been attributed to LND-mediated membrane damage, rather than to a direct interaction of the drug with the specific enzymes [6]. As part of the acquired information, relevant to the experiments reported in this paper, the activity of cytochrome *c* oxidase expressed by mitochondria isolated from Ehrlich ascites tumour cells were found not to be affected by treatment with LND [5].

Protocols suitable to single out, within the whole respiratory chain, the activity of cytochrome *c* oxidase most commonly include the use of specific inhibitors, such as antimycin, rotenone and/or myxothiazol [8]. In these cases reducing equivalents are commonly supplied to cytochrome oxidase through the redox couples ascorbate/TMPD, which are known to reduce cytochrome *c* [8, 9]. In this study the oxygen consumption activity of intact cardiac myocytes obtained from rats treated with LND have been examined.

Respiration was stimulated by ascorbate and TMPD according to recent experimental protocols that proved efficient in functionally isolating cytochrome oxidase in living myocytes [10]. The functional parameters and the electron microscopy patterns have been compared to those obtained using cardiac myocytes from untreated rats.

The evidence reported in this paper shows that previous interpretations, proposed by others to evaluate possible effects of LND on cytochrome *c* oxidase [5], may not be conclusive owing to a possible pitfall in the experimental design.

MATERIALS AND METHODS

Treatment of rats with LND. Sprague–Dawley male rats, 200–250 g in weight, were maintained under standard laboratory conditions, and fed for 4 weeks with a standard diet, including LND, 0.08% (w/w). Since the daily food consumption of the animals was on average 100 g/kg, the daily dose of LND was assumed to be ~80 mg/kg. The

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¶ Abbreviations: LND, lonidamine; TMPD, *N,N,N',N'*-tetramethyl-para-phenylendiamine.

concentration of LND was determined spectroscopically using the extinction coefficient calculated at 298 nm, $\varepsilon = 8\ mM^{-1}\ cm^{-1}$. The plasmatic saturation level of LND, 63 ± 15 μg/mL, was reached within 1 week of treatment commencing.

Cardiac myocytes were prepared according to Farmer *et al.* [11], following the protocol extensively described elsewhere [10]. Briefly, rats were killed by cervical dislocation; the excised heart was cannulated via the aorta and perfused, at 37°, with collagenase dissolved in Joklik-modified MEM supplemented with 10 mM HEPES buffer, pH 7.2. On average 2×10^7 cells per heart were collected.

The myocyte viability was assayed on the basis of: (1) functional and (2) morphological parameters. (1) Typically close to 90% of the cells were viable immediately after preparation, as judged from their ability to exclude Trypan Blue [12]. The number of viable cells decreased maximally to 70% within the experimental time, a few hours. (2) Damaged cells were more rounded in shape and displayed degenerative microscopic patterns, such as the presence of vacuoles and/or cytoplasmic granulations [11].

Cytochrome oxidase concentration was determined spectroscopically in intact cells using a photodiode array spectrophotometer [10]. Activity measurements were carried out polarographically, using a Clark-type oxygen electrode (Ysi Model 5300, Yellow Spring, OH, U.S.A.) equipped with a microvolume cell device (Model 600 A, Instech. Lab. Inc. Horsham, PA, U.S.A.). Typically, the assay was carried out in PBS Dulbecco's pH 7.2, supplemented with 1 mM $CaCl_2$, at 20°.

All chemicals used were of the highest degree of purity available. Lonidamine was kindly provided by Ist. Ricerca F. Augelini Sf A-Italy.

RESULTS

Following previous experimental protocols [10], the rate of oxygen consumption of myocytes was measured polarographically in the presence of excess ascorbate (10 mM) at different TMPD concentrations. As shown in Fig. 1, the oxygen consumption activity increased hyperbolically with the concentration of TMPD, up to a saturation level of $V = 40$ nmol O_2/min, when the myocytes were 1×10^4/mL and the number of cells equivalent to 10 nM cytochrome oxidase, as determined independently by spectroscopy. The rate of oxygen consumption of control and LND-treated myocytes was measured in the presence of saturating amount of TMPD (2 mM). The average oxygen consumption rate of cells prepared from control rats (N = 5) was $V_{O_2} = 40.5 \pm 5$ nmol/min, while the rate of myocytes prepared from LND-treated rats (N = 2) was $V_{O_2} = 68 \pm 4$ nmol/min. After normalization for cytochrome oxidase concentration the maximal turnover number of control and LND-treated cells was calculated to be $TN_{max} = 112$ and $189\ sec^{-1}$, respectively.

The activity of cytochrome oxidase of control and LND-treated cells was compared at two different concentrations of TMPD, namely 0.1 and 2 mM. Figure 2 shows the relative oxygraphic profiles for one such experiment. When the concentration of TMPD was 0.1 mM, the myocytes collected from control and LND-treated rats displayed the same rate of oxygen consumption, $V_{O_2} = 3.2$ nmol/min. At 2 mM TMPD, the oxygen consumption rate rose in both cases, though to a different extent: control myocytes $V_{O_2} = 44.6$ nmol/min, LND-treated $V_{O_2} = 72$ nmol/min; these rates were not enhanced by further addition of TMPD. The kinetics of oxygen consumption was zero order in all cases.

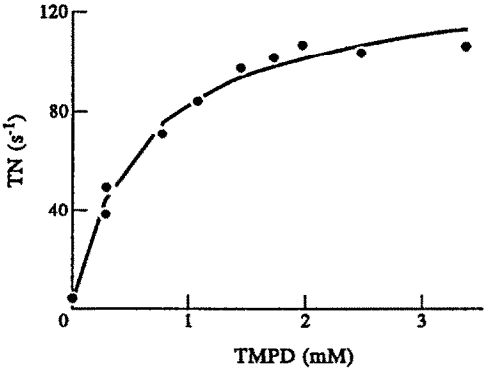

Fig. 1. Dependence of the oxygen consumption rate of cardiac myocytes on TMPD concentration. The rate of respiration expressed as turnover number, TN, was measured polarographically, using an oxygen electrode equipped with a microvolume (0.6 mL) cell-device. Assay medium: PBS Dulbecco's, pH 7.2, containing Ca^{2+} = 1 mM, Na/ascorbate = 10 mM; myocytes = 1×10^4/mL, equivalent to 10 nM cytochrome aa_3. $O_2 = 270$ μM. T = 20°. TN = $112\ s^{-1}$ is equivalent to 40 nmol O_2/min, consumed.

Immediately after heart perfusion, samples of myocytes were fixed using glutaraldehide and processed for electron microscopy observation (see Materials and Methods). Figure 3 shows two typical EM transmission patterns of LND-treated and control mitochondria. The mitochondria of control cells showed an orthodox shape, with dense matrix and transverse cristae. On the contrary, mitochondria of LND-treated myocytes were modified in shape with disarrangement of the cristae and evident alterations of the outer envelope membrane.

DISCUSSION

In the presence of excess of ascorbate and TMPD, the latter ensuring the rapid reduction of cytochrome *c*, cytochrome oxidase expressed its maximal activity and was functionally isolated from the rest of the respiratory chain [10]. Consistent with previous data [10], the concentration of TMPD necessary to fully stimulate the activity of cytochrome oxidase in myocytes, even in the presence of large excess ascorbate, was unexpectedly high. This finding could not be attributed to a low permeability of TMPD to the cytoplasmic or the external mitochondrial membrane, as treatment of the cells with digitonin had no effect [10]. Moreover, similar results have

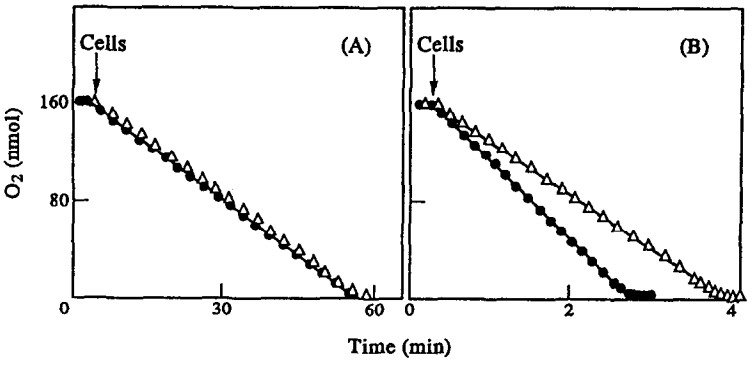

Fig. 2. Respiration profiles of cardiac myocytes at different reductant concentrations. Panel A: TMPD = 0.1 mM; panel B: TMPD = 2 mM. △, control; ●, LND-treated cells. All other conditions as in Fig. 1.

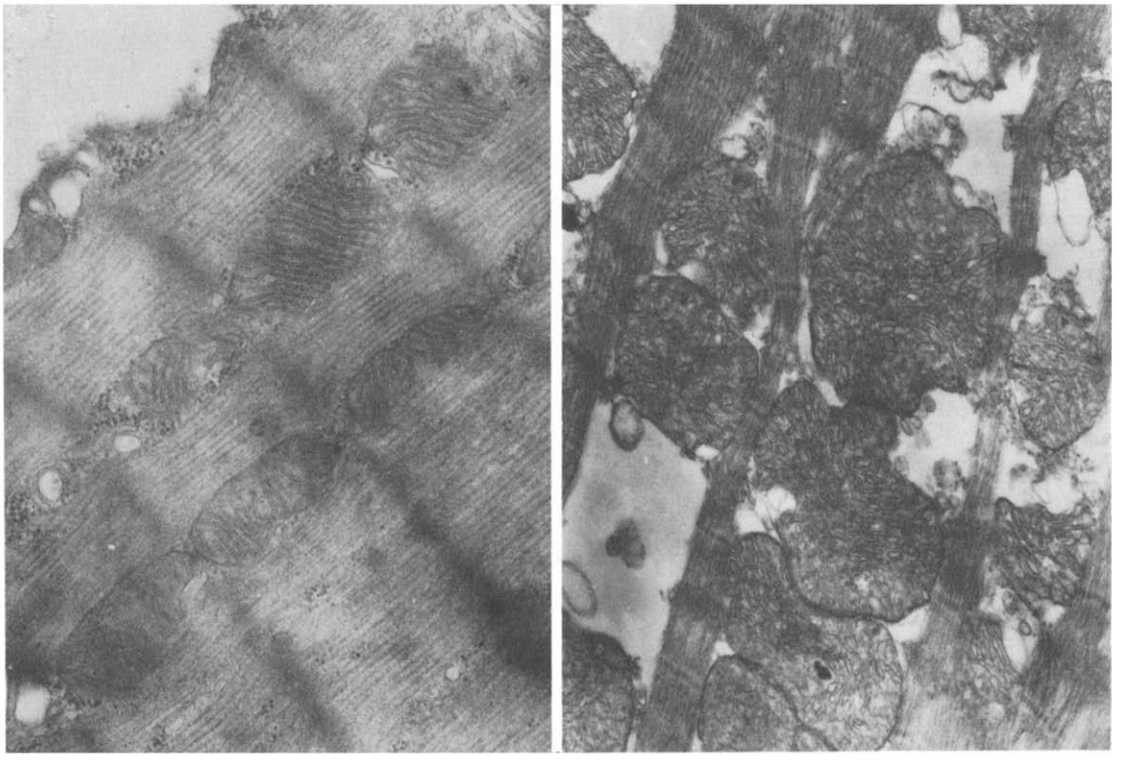

Fig. 3. Electron micrographs of control and LND-treated cardiac myocytes. Mitochondria of control cells (30,000×) showed an orthodox shape (left); mitochondria of treated rats (22,000×) showed evident modification of shape and structure (right), see text.

been reported by Morgan and Wikström [13] using rat liver mitochondria. These authors tentatively explained the high K_d value for TMPD, in the mM range, on the basis of aspecific TMPD–membrane interactions, lowering the activity of reduced TMPD in the intermembrane space of the mitochondrion.

Under physiological conditions, such as those used for these experiments, the reaction between cytochrome oxidase and molecular oxgen never limits turnover [14], as it is thermodynamically favoured, $K_m = 0.5\ \mu M$, and is very fast, the bimolecular rate constant being $k = 1 \times 10^8\ M^{-1}\ sec^{-1}$, at room temperature. Under the experimental conditions used it was assumed, at least to a first approximation, that the LND treatment did not affect the concentration of cytochrome *c*, the TMPD concentration may rather have been limiting the rate of oxygen consumption. Thus, the

dependance of the enzyme activity on TMPD concentration was studied. The reaction between TMPD and cytochrome oxidase, in the presence of saturating cytochrome *c* [14], can be described on the basis of the following minimal kinetic scheme:

$$a^{3+}a_3^{3+} \xleftarrow[\text{Step 1}]{K[\text{TMPD}]} a^{2+}a_3^{3+} \xrightarrow[\text{Step 2}]{\text{TN}_{max}} a^{2+}a_3^{2+}$$

where K is the apparent association equilibrium constant between reduced TMPD and the oxidized cytochrome aa_3, and TN_{max} is the turnover number directly reflecting the rate constant of the internal electron transfer from cytochrome *a* to cytochrome a_3. According to previous observations on cytochrome oxidase purified or reconstituted into phospholipid vesicles, TN_{max} reflects the slowest step in the catalytic cycle [15–17]. If step 1 is always at equilibrium relative to step 2 the observed turnover rate, i.e. TN_{obs}, will depend on the TMPD concentration. Within this approximation, and also according to [18], TN_{obs} is given by:

$$TN_{obs} = TN_{max} \frac{K[\text{TMPD}]}{K[\text{TMPD}] + 1}$$

which accounts for the dependence of turnover on TMPD concentration. When the TMPD concentration is small compared to K, TN_{obs} strongly depends on the TMPD concentration, and eventually, for [TMPD] approaching zero, the initial slope of the hyperbola in Fig. 1 approaches $dTN_{obs}/d[\text{TMPD}] = TN_{max}K$. At the saturating TMPD concentration $TN_{max} = 112\ sec^{-1}$, from the initial slope in Fig. 1, the phenomenological equilibrium constant for TMPD may be calculated as close to $K = 2 \times 10^3 M^{-1}$, a value consistent with other estimates reported in the literature [10, 13]. Myocytes prepared from control rat hearts, at a saturating concentration of TMPD, showed a maximal turnover rate of $V_{O_2} = 42$ nmol/min, a value close to that measured, under similar conditions, using myocytes obtained following similar rat heart perfusion protocols [10]. Treatment of rats with LND enhanced the oxygen consumption rate of myocytes, on average, by a factor of 1.6. This activation may tentatively be explained, taking the following into account: (1) LND has been proved to be highly soluble into phospholipid membranes [6]; (2) the molecular structure of LND, which is an hydrophobic heteropolycyclic carboxylic acid, shows structural similarities with compounds displaying protonophoric activity [5]; and (3) the administration of LND, according to previous observations [19] leads to modification of cell membranes, particularly of the mitochondrial membranes [3, 17, 18].

These observations taken together suggest that the accumulation of LND into the phospholipid bilayer enhances membrane permeability to ions, as proposed by others who showed drastic changes in membrane electrical properties of Ehrlich ascites tumour cells following treatment with LND [7]. A major consequence would be the (partial) loss of membrane impermeability, necessary to maintain the physiological level of the electrochemical potential gradient, $\Delta\tilde{\mu}_{H^+} = 200$ mV, used up by cells to drive ATP synthesis [8]. The proton electrochemical gradient is known to control cytochrome oxidase activity *in situ* [22] and after reconstitution of the enzyme into artificial phospholipid vesicles [23]. In the latter case it has been shown that, provided a suitable concentration of reducing substrates is available, the internal electron transfer from cytochrome *a* to cytochrome a_3, i.e. the rate limiting step in the catalytic cycle [16], is controlled by $\Delta\tilde{\mu}_{H^+}$ [18, 24]. Thus, a decrease of the $\Delta\tilde{\mu}_{H^+}$ value would be reflected in the rate limiting step and subsequently manifested as an increase in enzyme activity.

Consistently, after treatment with LND, which lowered $\Delta\tilde{\mu}_{H^+}$, an increase in the turnover rate was observed. This increase, however, was observed only at saturating concentrations of TMPD, since only under such conditions did the intrinsic activity of cytochrome oxidase limit the process. At lower TMPD concentrations (0.1 mM) the turnover rate was more than one order of magnitude slower, see also [10]: under such conditions, control and LND-treated cells behaved similarly. This was not unexpected, since at low concentrations of TMPD the rate of oxygen consumption was limited by the rate of reduction of cytochrome *c*. Consistently, no effect of LND on cytochrome oxidase activity was observed here, or by other authors [5].

The data reported here suggests that cardiac myocytes prepared from hearts of rats maintained on diets, including LND, undergo structural and functional changes of mitochondria compatible with those already described in cell cultures. Moreover, the role played by cytochrome oxidase with respect to the decrease of ATP synthesis has been better clarified. Within the limits of data obtained using a different experimental design, the authors believe that the LND-dependent enhancement of cytochrome oxidase activity escaped observation previously because the concentrations of TMPD used to stimulate respiration *in situ* were too low.

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