Modulation of Respiratory Function by 5,10,15,20-Tetrakis(N-methyl-4-pyridyl)Porphyrin-Zn(II) Tetrachloride in Isolated Rat Liver Mitochondria

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Mitochondria plays the central role in several degenerative and hyperproliferative pathologies and characterization of compounds able to enter mitochondria represents a highly targeted field for drug development. In the present paper we investigated the effects of a water-soluble porphyrin, 5,10,15,20-tetrakis(N-methyl-4-pyridyl)porphyrin-Zn(II)-tetrachloride (Zn-TNMPyP) on parameters of respiratory function in isolated rat liver mitochondria. Our data show that Zn-TNMPyP acts as a potent uncoupler of mitochondria with potential therapeutic effect.

Keywords: Zn-metalloporphyrin, rat liver mitochondria, respiratory parameters, uncoupling

In the recent years, due to the central role of mitochondria in several degenerative and hyperproliferative pathologies, development of compounds able to enter mitochondria, such as metalloprophyrins, represents a highly targeted field by the pharmaceutical industry [1-4]. Several therapeutic effects have been associated with the administration metalloprophyrins. Thus, zinc porphyrins have been reported to possess anti-inflammatory and anti-allergic properties in both *in vitro* and *in vivo* models by acting as an interleukin-1 (IL-1) antagonist or as an inhibitor of heme-enzymes [4-7]. Previous studies reported that a complex of porphyrin with Zn was able to inhibit reactive oxygen species (ROS) production induced by RAW264.7 macrophages in response to various stimuli, and was also effective in reducing oxidative stress-induced DNA-binding activity of NF-κB and IL-1 production [4,8]. Zinc protoporphyrin has also been demonstrated to inhibit mitogen-induced lymphocyte proliferation [9]. Zinc N-methyl-pyridyl-porphyrins at 10 micro M and 30 min of illumination were able to completely inhibit cell growth of human colon adenocarcinoma cell line [10].

5,10,15,20-tetrakis(N-methyl-4-pyridyl)porphyrin-Zn(II) tetrachloride (Zn-TNMPyP) is a water-soluble porphyrin (at variance from the vast majority of metalloporphyrins that are devoid of this property). Whether respiratory function of normal, healthy mitochondria is influenced in the presence of Zn-TNMPyP it is not known. Therefore, in the present study we thought to investigate the effect of Zn-metalloporphyrin on respiratory parameters in isolated rat liver mitochondria.

Experimental part

Materials and Methods

Adult male Sprague–Dawley rats (4–6 months, n = 10) were used. Animals were fed ad libitum and housed under standard conditions (constant temperature and humidity of 22.5 ± 2° C and 55 + 5%, 12-h light/dark cycle). Twenty-four hours prior to the experiment solid food was withdrawn with no limitation in water supply.

All experimental procedures used in this study were conducted in accordance with the Directive 2010/63/EU on the protection of animals used for scientific purposes. The experimental protocol was approved by the Committee for Ethics Research of "Victor Babes" University for Medicine and Pharmacy of Timişoara, Romania.

Reagents

All reagents used were of the highest quality available and were purchased from Sigma and Merck Chemicals. 5,10,15,20-tetrakis(N-methyl-4-pyridyl) porphyrin- Zn(II)-tetrachloride (Zn-TNMPyP) was synthetized and kindly provided by Eugenia FAGADAR-COSMA.

Mitochondria isolation

Rat liver mitochondria were isolated by differential centrifugation at 4°C, according to a previously described method [11]. Briefly, after animal anesthesia with an intraperitoneal injection of ketamine (Vetased, 30 mg/kg body mass) and xylazine (Xylazin, 10 mg/kg body mass), liver was quickly removed, cleansed of connective tissue, fat and blood and rinsed in ice-cold 0.9% KCl solution. Then it was minced with scissors to fragments smaller than 0.5 cm in a Petri dish and manually homogenized in a glass-teflon pestle homogenizer in 25 mL Buffer 1 (210 mM mannitol, 70 mM sucrose, 10 mM HEPES, 125 mg bovine serum albumin – BSA, 5 mg/mL and 0.25 ml EGTA 1 mM, *p*H = 7.4).

Mitochondria were isolated by differential centrifugation (Rotina 38R, Hettich) according to the following protocol: first centrifugation was performed at 750x*g* for 5 min at 4°C in order to remove the nuclei and unbroken cells which were pelleted by centrifugation. The supernatant, obtained by filtration, containing the mitochondrial fraction, was centrifuged at 7000x*g* for 10 min at 4°C. The resulting mitochondrial pellet was washed in Buffer 2 (210 mM mannitol, 70 mM sucrose, 10 mM HEPES, *p*H = 7.4). The final centrifugation was performed at 7000x*g* for 10 min at 4°C and the resulted mitochondrial pellet was gently

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 # Viorica Kerti and Dorina Coricovac have both the same contribution to this paper

resuspended in 0.25 mL Buffer 2 and kept on ice throughout the experiment. Protein concentration was determined by the Biuret method [12] after mitochondria solubilization with 1% deoxycolate, using BSA as standard.

Oxygen consumption measurements

Mitochondrial oxygen consumption was measured at 37°C using a Clark-type oxygen electrode (StrathKelvin 782 Oxygen System). All measurements were performed using mitochondria (1.0 mg mitochondrial protein/mL) in the incubation medium (100 mM KCl, 2 mM KH_2PO_4, 10 mM HEPES and 1 mM $MgCl_2$ *p*H=7.4). Oxygen consumption rates were expressed as natoms of O_2 consumed per minute and per mg of mitochondrial protein.

Basal respiration (State 2) was initiated after the addition of either complex I NAD-dependent substrates (glutamate 5 mM and malate 5 mM) or the complex II dependent-substrates (succinate 15 mM). In the latter case rotenone (1 mM) was added to inhibit complex I activity and to guarantee that the energy flux through the electron transport chain is controlled through $FADH_2$-linked substrates, i.e. succinate [13]. Active respiration (State 3) was measured after addition of ADP (3 mM). The intactness of the outer mitochondrial membrane was evaluated after addition of exogenous cytochrome c (32 μM). An increase of the respiratory rates after the exogenous addition of cytochrome c indicates the loss of endogenous cytochrome c due to the damage of outer mitochondrial membrane during the isolation process. Respiratory control ratio (RCR) was calculated as the ratio of State 3 and 2 respiratory rates and is correlated with the tightness of the coupling between mitochondria respiration and oxidative phosphorylation [14].

Three different concentrations (3, 5 and 10 μM) of Zn-TNMPyP were added to the incubation buffer and compared to the Control (non-treated) mitochondria.

Statistical analysis

The differences between groups were determined using one-way Anova and Tukey's and Bonfferoni's post hoc analysis. Results are presented as means ± SEM. Values for $p < 0.05$ were considered statistically significant.

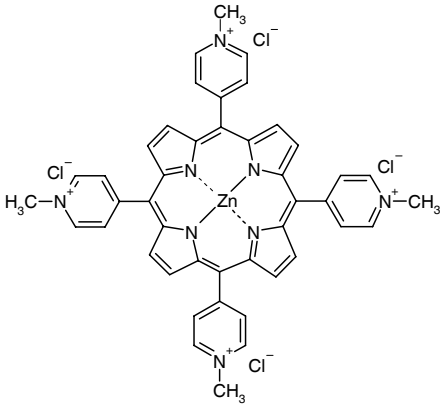

Fig. 1. Chemical structure of 5,10,15,20-tetrakis(N-methyl-4-pyridyl)porphyrin-Zn(II)-tetrachloride

Results and discussions

The precursor porphyrin base 5,10,15,20-tetrakis (4-pyridyl) porphyrin was synthetized and comprehensively characterized previously by NMR, UV-vis, FT-IR, fluorescence and thermal analysis [15] and by electrochemical investigations, respectively [16].

Meso-Tetrakis(N-methyl-4-pyridyl)porphine**,** that is a water soluble porphyrin, was synthesized according to literature data [17,18], by refluxing the hydrophobic precursor *meso*-tetra(pyridyl)porphyrin with an excess of methyl tosylate in *N*,*N*-dimethylformanide (DMF) for 8 hours. The tosylate counter-ion was ion-exchanged by Cl" for improving its solubility in water. Further classical metallation in water/DMF gives Zn-TNMPyP with amazing optoelectrical properties [19].

In the present study we have assessed the influence of Zn-TNMPyP on respiratory function in isolated rat heart mitochondria. A major advantage of this metalloporphyrin is its water solubility [18] that allowed the addition into the incubation medium in order to assess changes of State 2 (with substrates) or State 3 (with substrates and 3 mM ADP) respiration. We report a significant increase of both basal (State 2) and active respiration (State 3) in the presence of complex I-dependent substrates (glutamate/malate) after addition in the incubation medium of 5 μM of Zn-TNMPyP whereas the lowest concentration (3 μM) had no effect (fig.2). Interestingly, the highest concentration

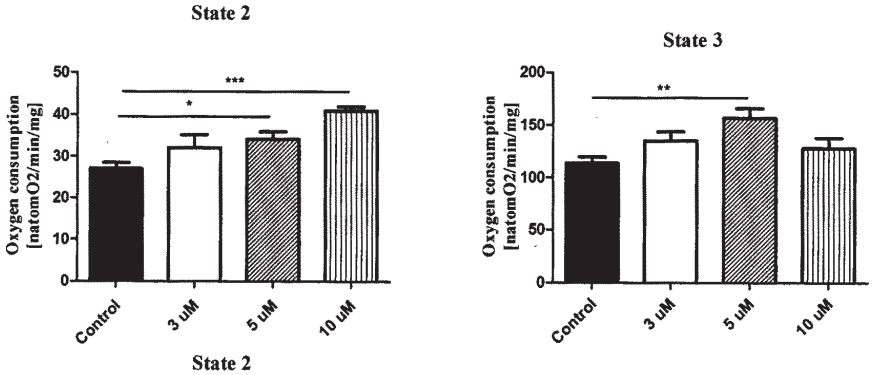

Fig. 2. Effects of Zn-TNMPyP on complex I-supported respiration

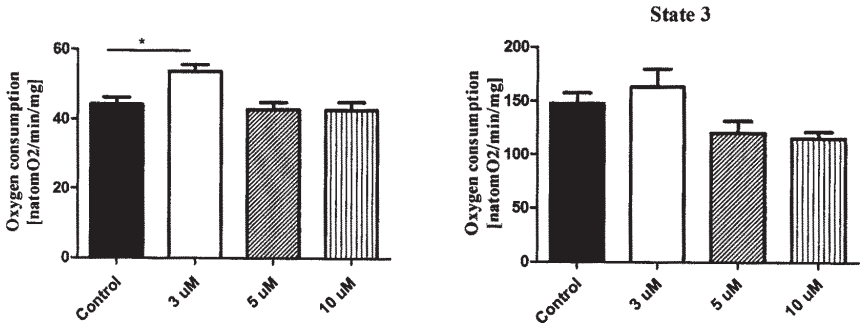

Fig. 3. Effects of Zn-TNMPyP on complex II-supported respiration

(10 μM) of the compound also induced a significant increase of State 2 respiratory rate but no further effect could be detected on ADP stimulated respiration (fig. 2). Regardless the concentration of Zn-TNMPyP, respiratory control ratio of mitochondria energized with glutamate and malate showed no significant changes (data not shown).

In the presence of complex II-dependent substrate (succinate + rotenone) a slight increase of basal respiration (State 2) after addition of 3 μM zinc porphyrin was observed; a similar trend was recorded for the active respiration (State 3) but it did not reach statistical significance (Fig. 3). Interestingly, no effects on respiratory parameters were elicited by the other two superior concentrations.

An increase in State 2 respiration demonstrates an uncoupling of mitochondrial respiration from oxidative phosphorylation (ATP synthesis) since changes in mitochondrial respiration rate in State 2 are proportional to proton flux through the mitochondrial inner membrane [20]. The most important uncoupling effect was recorded for the highest compound concentration (10 μM) in the presence of glutamate/malate as respiratory substrates, and for the lowest concentration (5 μM), when succinate was used, respectively. We have previously demonstrated that Zn-TNMPyP also uncoupled oxidation from phosphorylation in rat heart mitochondria; the effect was also present only for complex I-supported respiration at 5 μM concentration of the zinc porphyrin. Similarly to results in liver mitochondria, no effects on respiratory parameters were found for complex II-dependent respiration in heart mitochondria [21].

The observation that Zn-TNMPyP acts as a liver mitochondrial uncoupler is important since in a recent paper, treatment of high-fat fed rats that developed type 2 diabetes with a functional liver-targeted derivative of 2,4-dinitrophenol (a classical mitochondrial uncoupler) has been reported to safely reverse hypertriglyceridemia, fatty liver, hyperglycemia, and insulin resistance [22]. Further experiments are warranted in order to investigate whether our *in vitro* results can be recapitulated after the *in vivo* administration of the drug in intact and diseased animals.

Conclusions

We have demonstrated that 5,10,15,20-tetrakis(N-methyl-4-pyridyl)porphyrin-Zn(II) tetrachloride acts as a potent uncoupler of liver mitochondria. This observation is important in the light of the newly reported potential utility of liver-targeted mitochondrial uncoupling agents for the treatment of hypertriglyceridemia, nonalcoholic fatty liver disease, and metabolic syndrome.

Acknowledgments

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