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The effect of zinc acexamate on oxidative stress, inflammation and mitochondria induced apoptosis in rat model of renal warm ischemia

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ABSTRACT

Aim: Zinc has proved its efficacy in many models of ischemia reperfusion (I/R) injury. In this study, we used zinc acexamate (ZAC) as an exogenous source of zinc against renal I/R injury and we investigated whether its protective effects are mediated by the decrease of oxidative stress, inflammation, and mitochondria induced-apoptosis. Methods: Rats were orally pretreated with vehicle or ZAC (10 or 100 mg/kg) 24 h and 30 min prior to 1 h of bilateral renal warm ischemia and 2 h of reperfusion. Results: Our data showed that 10 mg/kg of ZAC, but not 100 mg/kg, improved renal architecture and function. Also, the low dose of ZAC up-regulated antioxidant enzymes activities and glutathione level and decreased lipids and proteins oxidation. Interestingly, the use of ZAC resulted in a significant reduce of pro-inflammatory cytokines (IL-1ß, IL-6 and MCP-1), enhanced mitochondria integrity and decreased expression of the pro-apoptotic protein caspase-9. Conclusion: We conclude that renal I/R induced oxidative stress, inflammation and apoptosis and that the use of ZAC at 10 mg/kg, but not 100 mg/kg, protects rat kidneys from I/R injury by down-regulating these processes.

1. Introduction

Renal ischemia/reperfusion (I/R) is one of the most common causes of acute renal infarct. It is associated with an increased risk of kidney dysfunction and rejection after renal transplantation [1]. In order to reduce I/R damage, different pharmacologic agents and surgical procedures have been investigated [2–6].

The pathophysiology of I/R injury is very complex and includes different mediators and cell signaling pathways which are interconnected. Oxidative stress has been shown to play a central role in renal I/R [7]. Indeed, under I/R conditions, large amounts of reactive oxygen species (ROS) are produced which make the cells unable to scavenge them. The accumulation of ROS contributes to lipid peroxidation, protein oxidation and DNA breakdown leading to organelle damages [8,9].

Relationship between inflammation and oxidative stress has been previously reported [10,11]. Inflammatory reaction is initiated by the release of various pro-inflammatory cytokines which activate effectors' cells such as macrophages, neutrophils and leucocytes leading to their migration and infiltration into the sites of inflammation. The activated cells produce large amounts of pro-inflammatory cytokines and ROS that lead to microcirculatory dysfunction and decrease of renal blood flow during the reperfusion phase [12].

Several studies revealed that ischemia causes mitochondrial alterations [13,14] and hinders oxidative phosphorylation mechanism and cell energy production [15]. In fact, during ischemia, oxidative stress and the accumulation of Ca^{2+} in the mitochondrial matrix could alter its membrane permeability and promotes the release of cytochrome-c into the cytoplasm which triggers apoptosis [16].

Zinc acexamate (ZAC) is a drug available in numerous countries worldwide. It has been used for the prevention of gastric and duodenal ulcers [17]. It has also been shown to have a potent-anabolic effect on bone which could prevent osteoporosis [18]. Moreover, ZAC plays a pivotal role on protecting against oxidative damage by attenuating lipid

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peroxidation [19], and this effect was related to the antioxidant capacity of its zinc (Zn) element. Indeed, Zn is able to induce metallothioneins [20], which are ROS scavengers [21]. Also, Zn is a component of superoxide dismutase (SOD), an antioxidant enzyme, which transforms superoxide ions into hydrogen peroxide [22]. Further, Zn decreased C-reactive protein, lipid peroxidation, and inflammatory cytokines in elderly subject [23].

The aim of the current study was to evaluate the effectiveness of ZAC, as an exogenous source of Zn, on regulating oxidative stress, inflammation and mitochondria-induced apoptosis in rat kidneys after I/R.

2. Material and methods

2.1. Animals

We used male Wistar rats weighing 200–250 g. Animals were maintained under standard conditions (temperature 23 ± 2 °C and 12 h of light-dark cycle) and had free access to water and food. Experimental protocol was conducted according to European Union regulations for animal experiments (Directive 86/609 CEE).

2.2. Surgical procedure

Rats were orally administrated with ZAC, dissolved in saline, 24 h and 30 min before ischemia using a gastric gavage probe. Rats were anaesthetized with an intra-peritoneal injection of pentobarbital (5%), and then they were subjected to surgery as described previously by Mahfoudh-Boussaid et al. [24]. Briefly, after abdominal midline incision, left and right renal pedicles were occluded for 1 h with smooth vascular clamps at 37 °C (warm ischemia). Reperfusion was initiated by removal of the clamps. At the end of reperfusion period (2 h or 24 h), blood samples were collected via a cannula inserted into the carotid artery. Simultaneously, tissues samples were collected and stored at −80 °C until analysis.

2.3. Experimental groups

Rats were randomly assigned to four groups, each containing 6 animals:

- Group I (Sham group): Rats were subjected to laparotomy and renal pedicles were dissected without occlusion.
- Group II (I/R group): Rats underwent 1 h of bilateral renal ischemia followed by 2 h of reperfusion.
- Group III (ZAC 10): Rats received ZAC solution (10 mg/kg body weight) orally 24 h and 30 min before ischemia. Then, rats were subjected to surgery as in group II.
- Group IV (ZAC 100): Same as in group III, but rats were treated with ZAC at 100 mg/kg of body weight.

2.4. Histopathological examination

Renal biopsies were fixed in 10% of formalin, embedded in paraffin, cut into 5 µm sections, and stained with standard hematoxylin-eosin. Histological evaluations were performed by an experienced pathologist who had no information about the treatment of groups by using a Leica light microscopy at a magnification of 200. A semi-quantitative analysis of histological renal damage was conducted according to the method of Jablonski et al. [25]. The score was from 0 to 4 as following: 0, normal; 1, necrosis of individual cells; 2, necrosis of all cells in adjacent proximal convoluted tubule (PCT), with survival of surrounding tubules; 3, necrosis confined to distal third PCT with bands of necrosis extending across inner cortex; 4, necrosis of all three segments of PCT.

2.5. Determination of lactate dehydrogenase activity

The activity of lactate dehydrogenase (LDH) was determined using commercial assay kit (Diagnostic System, Germany) according to manufacturer's instructions. It was expressed as U/l.

2.6. Measurement of creatinine concentration

Creatinine concentration was determined in plasma samples at 490 nm using the method of Jaffé (Spinreact kit, Spain) and expressed as µmol/l.

2.7. Determination of oxidative stress parameters

2.7.1. Lipid peroxidation assessment

Lipid peroxidation in renal tissues was evaluated by assessing malondialdehyde (MDA) and conjugated dienes (CD) formation. MDA level was measured at 530 nm using the thiobarbituric acid assay [26]. CD level was determined at 233 nm using a molar extinction coefficient of 25 200 $M^{-1}cm^{-1}$. They were expressed as nmol/mg of protein.

2.7.2. Measurement of reduced glutathione concentration

Reduced glutathione content (GSH) in renal homogenates was assessed by HPLC as previously reported [29]. Firstly, renal homogenates were mixed with TCA (10%) and centrifuged (maximum rpm, 1 min at 4 °C). Then, supernatants and standards were added with iodoacetic acid (100 mM) dissolved in 0.2 mM m-cresol. The pH of the obtained solution was adjusted to 9.0 ± 0.2 using a solution of (10 M) KOH/(3 M) $KHCO_3$. Samples and standards were incubated in the dark at room temperature for 30 min and then added with 1.5% of 1-fluoro-2,4-dinitrobenzene (DNFB). After that, they were incubated at 4 °C overnight. The next day, after sample centrifugation (13 000 g, RT, 1 min), N-DNP derivatives was injected into the loop of the HPLC system (Waters, Milford, MA, USA). Separation was achieved on a 3-aminopropyl column (5 µm; 4.6 mm × 20 cm; Custom LC, Houston, TX, USA). Initially, we used as solvents: solution A (80% grade HPLC methanol) and 20% solution B (0.5 M sodium acetate in 64% methanol), which were maintained for 10 min. From 10–40 min, we used a linear gradient, to a final ratio of 1% solution A/99% solution B. Eluted N-DNP derivatives were measured by ultraviolet detection at 365 nm.

2.7.3. Protein sulfhydryl content

Protein sulfhydryl (P-SH) content was determined using the method of Sedlak et al. [30]. This method consists of assessing total protein sulfhydryl groups (T-SH) and non-protein sulfhydryl groups (NP-SH) at 412 nm. The P-SH level was measured by subtracting the NP-SH from T-SH. It was expressed as mg/mg of protein.

2.7.4. Carbonyl protein concentration

Protein-bound carbonyls (CP) represent a marker of protein oxidation [31]. They were determined in renal tissue using two methods. In the first one, Ellman's reagent (10 mM DTNB) was prepared in 2.5 M HCl, according to the method of Vevine et al. [32]. Samples were mixed with the 2,4-dinitrophenylhydrazine (DNPH, 10 mM) for 1 h at room temperature. Then, they were centrifuged (4 000*g* for 5 min at 4 °C,) and the pellet was dissolved in 6.0 M of guanidine. Total proteins concentration was determined by the Bradford assay. CP content was measured at 370 nm using a molar extinction coefficient of 22 400 M^{-1} cm^{-1}. It was expressed as nmol/mg of protein.

We also measured the content of CP using an OxyBlot Protein Oxidation Detection kit (Chemicon, CA, USA). Renal tissues were homogenized in lysis buffer (Invitrogen) containing 50 mM DTT. Then, 5 µl of protein sample were added to 5 µl of 12% SDS. Samples were derivatized for 15 min at room temperature by adding 10 µl of DNPH solution and neutralized with 7.5 µl of neutralization solution. Protein samples were then loaded and separated into a polyacrylamide gel and

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