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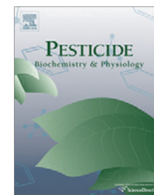

Hepatoprotective activity of angiotensin-converting enzyme (ACE) inhibitors, captopril and enalapril, against paraquat toxicity

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ABSTRACT

Paraquat is a highly toxic herbicide that is used in most countries without restriction. The cytotoxic action of paraquat is mediated by reactive radicals that are products of its metabolic reduction in cells. It has already been hypothesized that some angiotensin-converting enzyme inhibitors (e.g., captopril and enalapril) could show antioxidant and radical scavenging activity through their structural thiol groups, increasing antioxidant enzymes production or nitric oxide synthesis. In this study the hepatoprotective effect of captopril and enalapril against paraquat induced oxidative stress cytotoxicity was evaluated in isolated rat hepatocyte. Subtoxic concentrations of captopril (0.2 mM) and enalapril (0.2 mM) significantly ($p < 0.05$) protected the hepatocytes against paraquat (2 mM) induced oxidative stress cytotoxicity markers including: cell lysis, reactive oxygen species (ROS) generation, lipid peroxidation, glutathione depletion, mitochondrial membrane potential decrease, lysosomal membrane oxidative damage and cellular proteolysis. Moreover, we showed that non-thiol enalapril acts as well as thiol containing captopril at inhibiting oxidative stress cytotoxicity markers. Finally, our results support the hypothesis that it is the increase in nitric oxide synthesis and not the presence of the thiol group that accounts for the antioxidant activity of ACE inhibitors.

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1. Introduction

Paraquat (PQ), *N*,*N*′-dimethyl-4,4′-bipyridium is a widely used herbicide that causes severe lung and other damages in mammalians. This compound is very toxic to animals and humans with putative toxicity mechanisms associated with mitochondrial redox systems [1]. Generation of free radicals and oxidative damage to tissues leads to lipid peroxidation or NADPH depletion and collapse of the mitochondrial membrane potential by PQ [2]. PQ toxicity is related to the generation of the superoxide anion, which can lead to the formation of more toxic reactive oxygen species, e.g., hydrogen peroxide, often considered as the main toxicant [3]. It is noteworthy that there has not yet been any useful antidote for PQ toxicity in humans. Recently investigations showed that, inhibitors of the angiotensin-converting enzyme (ACE) had decreased pulmonary fibrosis caused by PQ in animal models [2,4,5].

Angiotensin-converting enzyme (ACE) inhibitors are commonly used in the treatment of hypertension and most forms of heart failures. The beneficial effects of ACE inhibitors were thought to be primarily due to the inhibition of angiotensin II formation [6]. Ghazi-Khansari et al. show that PQ lung toxicity can be prevented by chronic captopril and niacin treatment *in vivo* in mice [7]. The other work showed that enalapril or captopril treatments increase antioxidant enzymes and non-enzymatic antioxidant defenses in several mouse tissues [8]. Based on previous studies enalapril or captopril increased enzymatic (i.e., SOD) and non-enzymatic antioxidant defense systems in several mouse tissues [9]. It has also been suggested that captopril could prevent radiation-induced lung fibrosis in rats [10].

It has already been shown that increased reactive oxygen species (ROS) formation could cause oxidative damage to the cells, leading to several pathological conditions [11]. Previous studies also suggested that captopril could scavenge free radicals and limit superoxide generation, and modulate reactive oxygen and nitrogen species generation [12]. Tomita and collaborators reported that, when A549 cells (lung carcinoma cell line) were exposed to high concentrations of PQ (0.2 mM), increased levels of cytokines increased inducible NO synthase expression and nitrite accumula-

Abbreviations: PQ, paraquat; DMSO, dimethylsulfoxide; ROS, reactive oxygen species; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-(2-ethanesulfonic acid); OPT, O-phthalaldehyde; NEM, N-ethylmaleimide; LDH, lactate dehydrogenase; GSH and GSSG, reduced and oxidized glutathione; DCFH, 2′,7′-dichlorofluorescein diacetate; TBARs, thiobarbituric acid-reactive substances.

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tion in cells resulted in LDH release. They suggested that in PQ induced cell injury NO has both beneficial and deleterious actions [13]. Some other studies reported that nitric oxide synthase (NOS) is able to generate NO by oxidation of L-arginine. It was therefore postulated that PQ uses NOS as an electron source to generate superoxide anion ($O_2^{\cdot-}$) and therefore it decreases the generation of NO [13,14].

Due to suggested antioxidant and free radical scavenging activities mostly for thiol containing ACE inhibitors such as captopril, we planned to study hepatoprotective effects of captopril and enalapril against PQ induced oxidative stress cytotoxicity in isolated Sprague–Dawley rat hepatocytes using accelerated cytotoxicity mechanisms screening (ACMS) techniques [15].

Although, rat cellular metabolism is higher than human, but obtained results with rat hepatocytes can be extrapolated to human hepatocytes [16]. Our results supported protective effects of both thiol containing and non-thiol ACE inhibitors against PQ toxicity.

2. Materials and methods

2.1. Chemical

1-Bromoheptane, rhodamine 123 and hydrazine monohydrate were obtained from Aldrich Chemical Company (Milwaukee, WI, USA). Collagenase (from *Clostridium histolyticum*), bovine serum albumin (BSA) and Hepes (4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid) were purchased from Boehringer–Mannheim (Montreal, Canada). Trypan blue, paraquat dichloride salt, D-mannitol, dimethyl sulfoxide (DMSO), thiobarbituric acid (TBA), trichloroacetic acid (TCA), sodium pentobarbital and heparin and the other chemicals were obtained from Sigma (St. Louis, MO, USA). Acridine orange and dichlorofluorescin diacetate were purchased from Molecular Probes (Eugene, Ore, USA). All chemicals were of the highest commercial grade available.

2.2. Animals

This study was performed on 36 male Sprague–Dawley rats (weighing 280–300 g). They purchased from Institute Pasteur (Tehran, Iran) and were kept in individual cages in a controlled room (temperature, 20–25 °C humidity, 70–80%, exposed to 12 h of daylight). The rats were fed with standard rat food and tap water until experimentation. Twelve hours before the experiment feeding was stopped but the animals were allowed free access to tap water. Limitation of food and water was not applied to the animals that were put into their cages after the experiments. All experiments were conducted according to ethical standards and protocols approved by the Committee of Animal Experimentation of Shaheed Beheshti University of Medical Sciences, Tehran, Iran. The ethical standards were based on "European Convention for the Protection of Vertebrate Animals Used for Experimental and other Scientific Purposes" Acts of 1986, and the "Guiding Principles in the Use of Animals in Toxicology," adopted by the Society of Toxicology in 1989, for the acceptable use of experimental animals. All animals received humane care according to the criteria outlined in the "Guide for the Care and Use of Laboratory Animals" prepared by the National Academy of Sciences and published by the National Institutes of Health (NIH Publication 86-23, revised 1985).

2.3. Isolation and incubation of hepatocytes

Hepatocytes were obtained by collagenase perfusion of the liver [16]. Cells were suspended at a density of 10^6 cells/ml in round-bottomed flasks rotating in a water bath maintained at 37 °C in Krebs–Henseleit buffer (pH 7.4), supplemented with 12.5 mM Hepes under an atmosphere of 10% O_2, 85% N_2, and 5% CO_2.

2.4. Cell viability

The viability of isolated hepatocytes was assessed from the intactness of the plasma membrane as determined using trypan blue (0.2% w/v) exclusion test [17]. Aliquots of the hepatocyte incubate were taken at different time points during the 3 h incubation period. At least 75–80% of the control cells were still viable after 3 h.

2.5. Determination of reactive oxygen species

To determine the rate of ROS generation induced by PQ, dichlorofluorescin diacetate was added to the hepatocytes. DCFH penetrates in hepatocyte cells and then reacts with ROS to form the highly fluorescent dichlorofluorescein (DCF). The fluorescence intensity of DCF was measured using a Shimadzu RF5000U fluorescence spectrophotometer. Excitation and emission wavelengths were 500 and 520 nm, respectively. The results were expressed as fluorescent intensity per 10^6 cells [18].

2.6. Lipid peroxidation assay

Lipid peroxidation was determined by measuring the amount of thiobarbituric acid substances (TBARS) formed during the decomposition of lipid hydroperoxides by following the absorbance at 532 nm in a Beckman DU®-7 spectrophotometer [19].

2.7. Intracellular GSH and extra cellular GSSG assessment

GSH and GSSG were determined according to the spectrofluorometric method [20]. Each sample was measured in quarts cuvettes using a fluorimeter set for 350 nm excitation and 420 nm emission wavelengths.

2.8. Mitochondrial membrane potential assay

The capacity of mitochondria to take up the rhodamine 123 was calculated. The amount of rhodamine 123 remaining in the incubation medium was measured fluorimetrically using a Shimadzu RF5000U fluorescence spectrophotometer set at 490 nm excitation and 520 nm emission wavelengths [21].

2.9. Lysosomal membrane integrity assay

Hepatocyte lysosomal membrane stability was determined from the distribution of the fluorescent dye, acridine orange [16]. Aliquots of the cell suspension (0.5 ml) that were previously stained with acridine orange (5 μM) were separated from the incubation medium by 1 min centrifugation at 1000 rpm. The cell pellet was then resuspended in 2 ml of fresh incubation medium. This washing process was carried out for two times to remove the fluorescent dye from the media. Acridine orange redistribution in the cell suspension was then measured fluorimetrically using a Shimadzu RF5000U fluorescence spectrophotometer set at 495 nm excitation and 530 nm emission wavelengths.

2.10. Determination of proteolysis

Proteolysis was monitored using a fluorescence assay for tyrosine release [22]. An aliquot of the hepatocyte suspension was precipitated with an equal volume of 20% trichloroacetic acid and allowed to stand overnight at 4 °C. The sample was vortexed and centrifuged in a bench top clinical centrifuge (at 13,250 rpm for

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