Using Janus Green B to Study Paraquat Toxicity in Rat Liver Mitochondria

Role of ACE Inhibitors (Thiol and Nonthiol ACEi)

M. GHAZI-KHANSARI, a A. MOHAMMADI-BARDBORI, a, b
AND M-J. HOSSEINI a

a Department of Pharmacology, School of Medicine, Tehran University of Medical Sciences, Tehran, Iran
b Faculty of Pharmacy, Shiraz University of Medical Sciences, Shiraz, Iran.

ABSTRACT: Janus green B (JG-B) dye is used for vital staining of mitochondria and its reduction and oxidation shows the electron transfer chain alteration. The defect in electron transfer chain of mitochondria by paraquat is linked to free radical formation. In this present study we compared the abilities of different angiotensin-converting enzyme inhibitors, captopril (a thiol ACEi), enalapril, and lisinopril (two nonthiol ACEi) on mitochondria toxicity due to paraquat. The rat liver mitochondria were first isolated by centrifuge (at 4°C at a speed of 7,000 g) in a mixture of 0.25 M saccharose solution and 0.05 M Tris buffer. Various concentrations of paraquat (1, 5, 10 mM), enalapril (0.25, 0.5, 1 mM), lisinopril (0.01, 0.05, 0.1 mM), and captopril (0.08, 0.1, 1 mM) on the mitochondria isolated from the liver with respect to time were investigated. Paraquat at a concentration of 5 mM was determined to be significantly different compared to control values (P < 0.05) and captopril at a concentration of 0.08 mM, lisinopril (0.01 mM), and enalapril (0.25 mM) were found not to be significantly different from controls as found by spectroscopy at wavelength of 607 nm. Simultaneous treatment of mitochondria with captopril (0.08 mM) and paraquat (5 mM) significantly ameliorates the mitochondria toxicity of paraquat (5 mM) alone (P < 0.05). Our results show that captopril is a more effective antioxidant than the nonthiol ACEi. Lisinopril (0.01 mM) and enalapril (0.25 mM) did not significantly change the mitochondrial toxicity by paraquat (5 mM) (P > 0.05). The antioxidative
action of captopril appears to be attributable to the sulfahydryl group (SH) in the compound. This effect may be due to captopril’s abilities to scavenge reactive oxygen species.

**KEYWORDS:** paraquat; Janus green B; rat liver mitochondria; angiotensin-converting enzyme inhibitor (ACEi); ACE inhibitors (ACEi); rat liver

**INTRODUCTION**

Lewis (1923) used Janus green staining in a study on the role of the mitochondria in the development of the visual cells of chick embryos. JG-B has been used as a method for studying the oxidation-reduction potential of various tissues and organisms. It is probable that the cytochrome oxidase system plays a role in the super vital staining reaction. J-G B dye is prepared by conjugating diethyl safranine to dimethyl aniline through an azo linkage. Diethyl safranine is a red dye and JG-B is blue. Thus the conjugation with dimethyl aniline alters the resonating structure of the diethyl safranine and shifts the position of the absorption spectrum maximum toward the longer wavelengths with visible absorption spectrum maximum at 605 nm at pH 7. It has been shown that cytochrome c is reduced by leukosafranine and the leukosafranine is in turn oxidized to diethyl safranine. The cytochrome oxidase system, an enzyme within mitochondria, prevents the reduction of JG-B. This enzyme system is oxygen-dependent and cyanide-sensitive.

Paraquat (N, N’-dimethyl 4, 4’-bipyridium) is very toxic to animals, including humans, with putative toxicity mechanisms associated with mitochondrial redox systems. Mitochondria are candidate targets of paraquat toxicity in animal tissues and plants. The mechanisms of paraquat toxicity are frequently related to the generation of the superoxide anion, which can lead to the formation of more toxic reactive oxygen species, for example, hydrogen peroxide, often taken as the main toxicant. Palmeira et al. reported that paraquat at a dose of 1mM, through its effect on uncoupling system, causes respiratory system depression by means of inhibition of mitochondrial complexes (I) and (V).

Angiotensin-converting enzyme (ACE) inhibitors, which have both an antihypertensive and a cardioprotective action, are commonly used in the treatment of hypertension and most forms of heart failure. The beneficial effects of ACE inhibitors were thought to be primarily due to the inhibition of angiotensin II formation. However, a number of studies have shown improvement of oxidant stress and fibrosis. Treatment with enalapril or captopril was shown to increase antioxidant enzymes and nonenzymatic antioxidant defenses in several mouse tissues.

In this report, we studied the role of three ACE inhibitors—captopril, enalapril, and lisinopril—to protect against mitochondria toxicity by paraquat.
MATERIALS AND METHODS

Compounds

Paraquat dichloride salt, captopril, lisinopril, and enalapril were purchased from the Sigma Chemical Co. (St. Louis, MO, USA). Janus green was obtained from Merck (Germany). All other chemicals were obtained from the Sigma Chemical Co.

Animals and Experimental Groups

This study was performed on 36 male Wister albino rats (weighing 80–120 g). They were kept in individual cages in a controlled room (temperature, 20–25°C; humidity, 70% to 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 the rats were stopped from feeding, but 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.

Preparation of Mitochondria

The liver was removed and minced with small scissors in a cold manitol solution containing 0.225 M D-manitol, 75 mM sucrose, and 0.2 mM ethylenediaminetetraacetic acid (EDTA). The minced liver (30 g) was gently homogenized in a glass homogenizer with a Teflon pestle and then centrifuged at 700 \( \times g \) for 10 min at 4°C to remove nuclei, unbroken cells, and other non-subcellular tissue. The supernatants were centrifuged at 7,000 \( \times g \) for 20 min. These second supernatants were pooled as the crude microsomal fraction and the pale loose upper layer, which was rich in swollen or broken mitochondria, lysosomes, and some microsomes, of sediments was washed away. The dark packed lower layer (heavy mitochondrial fraction) was resuspended in the manitol solution and recentrifuged twice at 7,000 \( \times g \) for 20 min. The heavy mitochondrial sediments were suspended in Tris solution containing 0.05 M Tris-HCl buffer (pH 7.4), 0.25 M sucrose, 20 mM KCl, 2.0 mM MgCl\(_2\), and 1.0 mM Na\(_2\)HPO\(_4\) at 4°C before assay.

Mitochondrial Staining Procedure

A 1.0 mL of mitochondrial (10 mg protein) suspension treated with different concentrations of paraquat and ACEi were added with 1mL of JG-B (1 ppm). The blank solution was prepared in the same way without addition of mitochondrial suspension. Each sample was then measured via spectrophotometry at 607 nm within 40 min at every 10-min interval.
Viability Percentage Calculation

The percentage of mitochondrial viability of each test sample was calculated as described by Heidari et al. (2001) as follows:\textsuperscript{15,16} 

\[
\% \text{Mitochondrial toxicity} = 1 - \frac{\text{Mean absorbance of toxicant}}{\text{Mean absorbance of negative control}} \times 100
\]

\[
\% \text{viability} = 100 - \% \text{Mitochondrial toxicity}
\]

Experimental Design

Mitochondria suspension was divided into 13 groups for dose–response determination. Group 1 contained control, groups 2–4 contained enalapril (0.25, 0.5, 1 mM), groups 5–7 contained captopril (0.1, 0.08, 1 mM), groups 8–10 contained lisinopril (0.01, 0.05, 0.1 mM), and groups 11–13 contained paraquat (1, 5, 10 mM).

For treatment study, mitochondria suspension was divided into eight groups. Group 1 contained control, group 2 contained paraquat only (5 mM),

![FIGURE 1. Effect of different concentrations of captopril (0.1, 0.08, 1 mM) on percentage viabilities of rat liver mitochondria suspension. There was no significant decrease in viability at 0.08 mM captopril dose levels as compared to control groups. All data are given as mean ± SEM, 3–4 mitochondria suspensions per group.](image)
group 3–5 contained enalapril (0.25 mM) or captopril (0.08 mM) or lisinopril (0.01 mM), and group 5–8 contained paraquat (5 mM) together with enalapril (0.25 mM) or captopril (0.08 mM) or lisinopril (0.01 mM).

**Statistical Analysis**

All values were expressed as mean ± standard error (SEM) of 3–4 samples. Analysis of variance (ANOVA) followed by Student–Newman–Keuls test was used to evaluate the significance of the results obtained. All computations were analyzed by computer using SPSS software.

**RESULTS**

*Dose Response of Angiotensin-Converting Enzyme Inhibitors and Paraquat*

Angiotensin-converting enzyme inhibitors were shown to decrease viability dose-dependently (Figs. 1–3). Enalapril at a dose of 0.25 mM, captopril at a...
FIGURE 3. Effect of different concentrations of enalapril (0.25, 0.5, 1 mM) on percentage viabilities of rat liver mitochondria suspension. There was no significant decrease in viability at 0.25 mM enalapril as compared to control groups. All data are given as mean ± SEM, 3–4 mitochondria suspensions per group.

dose of 0.08 mM, and lisinopril at a dose of 0.01 mM were shown not to be significantly different from control ($P > 0.05$). Therefore, the above doses were used for the treatment study. Paraquat at a concentration of 5 mM (Fig. 4) was determined to be significantly different compared to control ($P < 0.05$).

**Effect of Angiotensin-Converting Enzyme Inhibitors on Mitochondria Toxicity of Paraquat**

Simultaneous treatment of mitochondria with captopril (0.08 mM) and paraquat (5 mM) (Fig. 5A) significantly ameliorates the mitochondria toxicity of paraquat (5 mM) alone ($P < 0.05$). Lisinopril (0.01 mM) (Fig. 5B) and enalapril (0.25 mM) (Fig. 5C) did not significantly change the mitochondrial toxicity by paraquat (5 mM) ($P > 0.05$).

**DISCUSSION**

There are two suggested mechanisms for paraquat mitochondrial toxicity.
FIGURE 4. Effect of different concentrations of paraquat (1, 5, 10 mM) on percentage viabilities of rat liver mitochondria suspension. There was a significant decrease in viability at 5 mM paraquat as compared with control groups. All data are given as mean ± SEM, 3–4 mitochondria suspensions per group.

Alternate Electron Acceptors

Alternate electron acceptors are substances capable of extracting electrons from intermediates in the respiratory chain, competing with the natural substrates. These substances may also affect redox cycle, passing electrons back to the respiratory chain at a later point, bypassing sites in the chain essential for energy generation.

Inhibition of the Respiratory Chain

The respiratory chain can be inhibited at any of the four protein complexes in the respiratory chain, although effects on complex IV (cytochrome c oxidase) are the most severe because this is the step where oxygen is reduced to water. Inhibition at complex III can result in the generation of reactive oxygen species as the consequence of the inherent instability of the electron transfer process to this complex from reduced ubiquinone. JG-B in this study was not only used for vital staining of mitochondria, but also shows the alteration of the
FIGURE 5. Effect of captopril (0.08 mM), lisinopril (0.01 mM), and enalapril (0.25 mM) on paraquat toxicity (5 mM) isolated rat liver mitochondria. All dose levels compared with control groups. All data are given as mean ± SEM, 3–4 mitochondria suspensions per group.
electron transfer chain in mitochondria. Since paraquat also affects the electron transfer chain, JG-B is a good marker for its toxicity. FIGURE 4 shows paraquat dose-dependently increased the mitochondria toxicity. FIGURE 1–5 also shows that JG-B is reliable to study the effect of ACEi on paraquat mitochondria toxicities.

We found significant differences among three assayed ACEi in mitochondria toxicity induced by paraquat (FIG. 5A, B, C). Captopril was the most effective in decreasing mitochondria toxicity by paraquat. Our observation suggests that the presence of a thiol group in the ACEi structure may be a determinant for the antioxidant properties. Under normal physiological conditions, antioxidants protect against reactive oxygen species (ROS) generated in the mitochondria. Numerous past studies have employed antioxidants, such as vitamin E and melatonin, as treatments for excessive ROS production. Captopril was shown to partially prevent the decrease of coenzyme Q10 level, dimension of cytochrome oxidase activity state 3, oxidative phosphorylation rate (OPR), and the enhancement of mitochondrial F1ATPase protein concentration. CoQ10 transfers electrons from complex I and II to complex III, and stabilizes respiratory complexes at the level of the inner mitochondrial membrane. It seems that ACEi may protect tissues from oxidative damage by increased mitochondrial coenzyme Q10 level, improved respiratory chain function, and energy production. These effects may be due to captopril’s abilities to scavenge reactive oxygen species as evaluated in vitro by Janus green B, a vital marker of mitochondria.

Of the angiotensin-converting enzyme inhibitor we used only pretreatment with thiols containing compounds (captopril) that were able to quench ROS generation from isolated mitochondria. Our data demonstrate that treatment with captopril can prevent damage induced by paraquat and therefore may be considered a therapeutic agent in the prevention and treatment of environmental toxins such as paraquat. Our results also suggest that JG-B dye is a simple, rapid, safe, cost-effective, reproducible, and reliable method to study mitochondria toxicity.

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REFERENCES


