Hepatoprotective role of captopril on paraquat induced hepatotoxicity

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Paraquat (PQ) is a highly toxic herbicide that is used in most of the countries without restriction. The cytotoxic effect of PQ is mediated by radicals, which are the products of PQ reduction in cells. The anti-oxidative action of captopril, an angiotensin-converting enzyme inhibitor, appears to be through its ability to scavenge reactive oxygen species. In this study, the hepatoprotective effect of captopril against PQ-induced hepatotoxicity was evaluated using primary cultured rat hepatocytes. Hepatocytes were isolated from male Wistar rats using a two-step collagenase perfusion, following incubation in the presence of captopril at 0.1, 0.2, 0.4 and 0.8 mM with or without PQ (5 mM). Hepatoprotective effects of captopril were studied indicating glutathione level intensity, triobarbituric acid reactive substances (TBARs) formation, lactate dehydrogenase (LDH) leakage and cell viability every 70 min for 210 min. Captopril at 0.2 mM concentration maintained the LDH leakage, glutathione level and cell viability in the presence of 5 mM PQ. In spite of a significant elevation in TBARs formation in the PQ group, captopril did not show any significant protection. In conclusion, our data reveals that incubation of freshly isolated rat hepatocytes with captopril (0.2 mM) significantly protected the hepatocytes against the cytotoxicity of PQ (P < 0.05). *Correspondence: Ghazi-Khansari M, PhD, Associate Professor, Department of Pharmacology, School of Medicine, Tehran University of Medical Sciences, PO Box 13145-784, Tehran, Iran E-mail: ghazikha@sina.tums.ac.ir; khansagm@yahoo.com

Introduction

The bipyridyl herbicides such as paraquat (PQ) are widely used to control the unwanted vegetation. Paraquat (1,1’-dimethyl-4,4’-bipyridylidium dichloride) causes acute cell damage by redox cycling. Consequent cell injury demands experiments to evaluate the toxicity mechanisms exerted on cell metabolism.1,2

Paraquat is converted to free radicals, which react rapidly with dioxygen and converts it to \( O^2- \). The oxidative stress caused by the extensive amounts of \( O^2- \), depletes cellular GSH (gamma-L-glutamyl-L-cysteinylglycine) resulting in an increased susceptibility of the hepatocytes to membrane lipid peroxidation.1 The peroxidation of polyunsaturated fatty acids in the membrane lipid is considered to be a basic mechanism for chemical toxicity leading to cellular membrane damage and consequently lysosomal enzymes release.3 The membrane injury may also cause lactate dehydrogenase (LDH) leakage, which is a well-known indicator of cell membrane integrity and cell viability.4

Angiotensin-converting enzyme (ACE) inhibitors are popular drugs in the treatment of hypertension and congestive heart failure.5 Other pharmacological effects such as free radical scavenger action, reduction of oxidant stress6 and anti-fibrotic effects have been postulated.7

It has been shown that captopril, a prototype ACE inhibitor, increases the activity of liver superoxide dismutase and glutathione peroxidase, which are of the main anti-oxidant enzymes found in aerobic organisms, in vitro independently of ACE inhibition. This activity protects cells from oxidative damage, although the mechanism is not fully understood.5

Paraquat, when ingested accidentally or intentionally accumulates in high concentrations in kidney, liver and lung resulting in damage to these organs and consequently death occurs within one week because of multiple organ failure.8 High mortality rate in these patients imposes a challenge to clinical practitioners. As recent animal and clinical studies have demonstrated apparent protection of early administration of free radical scavengers and anti-inflammatory therapy, awarding to PQ-intoxicated lungs, corresponding management of hepatotoxicity of PQ considering extrapulmonary effects of the toxin is mandatory.6 Protective protocols for acute
hepatocyte injury in an in vitro experiment can be a promising step to this clinical achievement during management of PQ poisoning.

Freshly isolated rat hepatocyte provides a useful system for the study of temporary processes leading to cell damage caused by drugs and chemicals. In this study the effects of captopril on PQ-induced GSH depletion, lipid peroxidation and LDH leakage, were studied in isolated rat hepatocytes.

Materials and methods

Animals
Male Wistar rats (200–250 g) were housed environmentally (t = 25°C) in air humidity controlled room (60%), which kept on standard laboratory diet and were maintained on a 12 h light–dark cycle and starved overnight before hepatocyte isolation. The Animal Ethics Committee of the Tehran University of Medical Sciences, School of Medicine, Education Section of Basic Sciences approved all of experiments (210/20021, 1 July 2000).

Chemicals
The chemicals were obtained from the following sources. Collagenase type B was purchased from Roche Company, Germany. Ethylene glycol-O, O’-bis (2-aminomethyl)-N,N’,N”-tetraacetic acid, 5,5’-dithio-bis-(2-nitrobenzoic acid) (DTNB), Thiobarbitoric acid (TBA) and Glutathione (GSH) were obtained from Fluka Biochemika Co. (Switzerland). N-2-hydroxyethyl piperazine-N’-2-ethanesulfonine acid, Tris (hydroxy methyl) aminomethane, captopril and Methyl viologen (PQ) from Sigma-Aldrich Chemie Gmbh, Germany. Lactate Dehydrogenase kit was from Zist Shimi Co., Tehran, Iran. Krebs-Henseleite buffer materials (NaCl, MgSO4·7H2O, KCl, NaHCO3, Na2HPO4·7H2O and Glucose) were purchased from Merck Chemical Co.

Hepatocyte isolation
Rat hepatocytes were isolated by a two-step collagenase perfusion as described elsewhere. The viability of the hepatocytes obtained was usually 85% as determined by trypan blue exclusion. Freshly isolated rat hepatocytes (1.5–2 × 10^6 cells/mL) were incubated in Krebs-Henseleite buffer (pH = 7.4) containing 1.5% (W/V) BSA.

The cells were incubated in Erlenmeyer flasks in a temperature controlled rotary shaker (110 rpm) for 20 min to be stabilized then each concentration of captopril (0.1, 0.2, 0.4 and 0.8 mM) was added 15 min before PQ addition (5 mM). Samples were taken every 70 min in order to measure viability, GSH, lipid peroxidation (LPO) levels and percentage of LDH leakage.

Total glutathione (GSH) determination
Glutathione was determined according to Ellman method, with some modification. One millilitre mL sample of cell suspension was taken at the designated intervals and washed three times with Krebs-Henseleite buffer and then centrifuged at 10 000 RPM for 30 min. Five-hundred microtitre of TCA (10%) and 300 μL of double distilled water were added to the pellet and the mixture was centrifuged after 30 min. Seven-hundred microtitre of supernatant was mixed with 875 μL of Tris buffer (1M) and 200 μL of DTNB (0.04%). The absorbance of the mixture was determined at 412 nm.

Thiobarbituric acid assay for lipid peroxidation
Malonaldehyde has been identified as the product of lipid peroxidation (LPO), which can be monitored by TBA assay. Buege and Aust method was used to assess TBA reactive substances. In brief 0.5 mL of hepatocyte suspension was mixed with 0.5 mL TCA (15%) followed by 3000 RPM × 10 min centrifugation. About 2.0 mL of TCA–TBA–HCl stock reagent containing 15% TCA, 0.375% TBA and 0.25 N HCl was combined with the pellet. The solution was heated for 15 min in a boiling water bath and then cooled. The flocculent precipitate was obtained by centrifugation at 1000 g for 10 min. The absorbance of the supernatant at 535 nm was measured.

Lactate dehydrogenase (LDH) leakages
Levels of LDH leakage was measured by UV/VIS spectrophotometry at 340 nm using commercial kit of Zist Chemi (Tehran-Iran). The LDH leakage was expressed as percentage of extracellular leakage to the total LDH activity present in the cells. The anti-hepatotoxicant potential of captopril was calculated by the extent of LDH leakage compared with PQ leakage alone.

Statistical analyses
Statistical significance of differences between means of groups was evaluated using ANOVA followed by Tukey test with SPSS software. A value of P < 0.05 was considered significant.

Results

Viability
Addition of PQ (5 mM) to the isolated rat hepatocytes results in a time-dependent cell death. Cell death, in terms of trypan blue exclusion, increased...
significantly after 70 min. Captopril (0.2 mM) in comparison with the other concentrations (0.1, 0.4, and 0.8 mM) significantly prevented the hepatocellular death induced by PQ ($P < 0.01$) (Figures 1A and 2A).

*Lactate dehydrogenase*

About 5 mM PQ was found to be cytotoxic in freshly isolated hepatocytes resulting in significant increase in LDH leakage. Captopril (0.2 mM) maintained LDH leakage close to control levels ($P < 0.01$) (Figures 1B and 2B).

*Glutathione status and TBARs formation*

Incubation of fresh hepatocytes with herbicide PQ leads to a significant decline in intracellular GSH after 210 min ($P < 0.01$). Treatment with captopril at the concentration of 0.2 mM made a substantial effect on GSH depletion caused by PQ, versus the cells treated with PQ alone (Figures 1C and 2C).

The amount of TBARs increased in hepatocyte suspension in the presence of 5 mM PQ ($P < 0.05$). Captopril at concentration of 0.2 mM had no significant effect on LPO induced by PQ (Figure 3).

**Discussion**

In the present study using an *in vitro* model of PQ toxicity, we showed that captopril is effective in reduction of PQ hepatotoxicity in freshly isolated rat hepatocytes. Pretreatment with captopril (0.2 mM) prevented hepatocellular death and LDH leakage caused by PQ.

Current approaches in the management of PQ poisoning mainly consider either modification of its toxicokinetics or reduction of the induced inflammation. Unfortunately, these approaches usually fail to change the fatal outcome. However, various studies have described PQ-induced lung toxicity and some preventive protocols have been introduced, no effective therapy has been developed for hepatotoxicity of PQ. As oxidative damage is the key issue in PQ toxicity, anti-oxidant therapy can be theoretically feasible.

Glutathione and LPO are both sensitive indicators of oxidative stress. Glutathione involves in many aspects of cellular metabolism and regulation, playing a crucial role in the cellular anti-oxidant defense system by scavenging free radicals. Infact, it can prevent oxidation of biomolecules by removing hydrogen and lipid peroxides. Aydin *et al.* indicated that LPQ, as measured by TBARS, is an index of malonaldehyde production and has been implicated in a number of deleterious effects such as increased membrane rigidity, osmotic fragility, decreased cellular and subcellular components, reduced mitochondrial survival and lipid fluidity.16

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**Figure 1** Effects of different concentrations of captopril on paraquat-induced cytotoxicity after 210 min, cell viability (A), LDH leakage (B), GSH depletion (C). Data are mean ± SEM of 3–5 measurements.

†$P < 0.05$, ††$P < 0.01$, †††$P < 0.0001$ significantly different when compared with control.

$*P < 0.05$, **$P < 0.01$ significantly different when compared with paraquat group.
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peroxide and mainly the dioxygen superoxide anion radical \( \text{O}_2^- \). Peroxidation products react with lipoprotein in the cell membrane and leading to hepatocyte injury.\(^1\) Some investigators have suggested that PQ produces its toxic effects by inducing membrane LPO.\(^14\) In addition it was shown that the hepatotoxicity induced by the herbicide is mediated by processes involving GSH oxidation.\(^1\)

Intracellular reduced GSH plays an important role in the detoxification of variety of xenobiotics and during this reaction, glutathione disulfide is formed. Angiotensin-converting enzyme inhibitors are considered a rather safe group of therapeutic agent with no serious side effects. Captopril inhibits the ACE that catalyses the conversion of angiotensin I to the vasoconstrictor peptide, angiotensin II. It is generally recommended for the treatment of hypertension, congestive heart failure, acute myocardial infarction and renal complications of diabetes mellitus. It also has beneficial experimental effects in hindering the progression of chronic renal failure, diabetic nephropathy and development of atherosclerosis.\(^17\)–\(^19\) Also there is increasing evidence that the broader pharmacological properties of ACE inhibitors encompass the anti-oxidant ability through scavenging free radical because of its terminal-SH group in a variety of organ systems.\(^6\),\(^20\)–\(^22\) Previously, we have shown that captopril ameliorates the biochemical alterations caused by PQ in isolated perfused rat lung.\(^6\) Pulmonary ACE activity significantly reduces after PQ treatment. Therefore, captopril may reduce the toxicity of PQ through other mechanism.

The anti-oxidant activity of captopril possibly ameliorates the oxidative stress. The -SH group in

Figure 2  Time course effects of captopril (0.2 mM) on paraquat-induced cytotoxicity, cell viability (A), paraquat-induced LDH leakage (B), paraquat-induced GSH depletion (C). Data are mean ±SEM of 3–5 measurements.

\(^{**}P < 0.01\), \(^{***}P < 0.001\) significantly different when compared with control.

\(^{*}P < 0.05\), \(^{**}P < 0.01\) significantly different when compared with paraquat group.

High levels of PQ are converted by one electron reduction to free radicals, which react very rapidly with dioxygen. This reaction regenerates the native bipyridyl, converts the hydroxyl radicals, hydrogen

Figure 3  Effect of captopril (0.2 mM) pretreatment on paraquat-induced lipid peroxidation after 210 min. Data are mean ±SEM of 3–5 measurements.

\(^{*}P < 0.05\) significantly different when compared with control group.
the structure is a crucial requirement for free radical scavenging activity but not the proline part.\textsuperscript{23} Our data revealed that restoration of GSH levels following captopril pretreatment might be related to the -SH group leading to higher GSH level, strengthening the anti-oxidant defense in the hepatocytes. Moreover, the reactive oxygen species, produced during PQ treatment, may be directly detoxified by \(-\text{SH}\) in captopril itself.\textsuperscript{24} These results are consistent with the findings of Chopra et al.\textsuperscript{25} demonstrating that the sulphydryl group in the ACE inhibitors might behave similar to \(-\text{SH}\) group in GSH.

Lipid peroxidation induced by reduction of GSH generates reactive aldehydes that may react with protein thiols.\textsuperscript{26} Lipid peroxidation and the injury induced by PQ affect membrane integrity, leading to LDH leakage as this was demonstrated by increase in LDH index in this study. These data suggest that peroxidation products may react mainly with captopril rather than lipoprotein in the cell membrane, thereby preventing oxidation of the structure. The decreased LDH leakage indicates the reinstated homeostasis of the hepatocytes following captopril treatment, resulting in stable cell membrane, as it was shown in isolated perfused rat lung.\textsuperscript{6} This may highlight the membrane interaction.

There are reports presenting that reactive oxygen species mainly attack cell membrane lipids, so LPO is the consequence. Chopra et al.\textsuperscript{25} showed that ACE inhibitors hinder LPO in microsomes independent of the proline part. Moreover, it was indicated that captopril can decrease LPO and superoxide dismutase activity and hydroxyproline level, also it inhibits reduction in glutathione peroxidase activity and GSH levels in the lung tissue following PQ administration.\textsuperscript{27} Other reports have shown that the \(-\text{SH}\) compounds can themselves stimulate LPO through promoting \(\text{Fe}^{3+}/\text{Cu}^{2+}\)-mediated peroxidation.\textsuperscript{25} Our results suggest that captopril in the concentration that we used cannot attenuate the acute PQ-induced LPO at least in freshly isolated hepatocyte, however, the GSH is restored. These conflicting results can be confronted with the possible different pathways used by PQ to initiate and augment LPO in the membrane of hepatocytes.

A recent study has investigated the effects of three stress models (immobilization, cold and immobilization–cold) on protein oxidation, LPO and the anti-oxidant defense system in peripheral tissues (liver, kidney and heart), concluding that the most affected tissue was that of heart with regard to level of TBARs because of its highest mass-specific oxygen consumption.\textsuperscript{28} The results of this study may indicate that lower oxygen consumption in liver contributes to different pattern of LPO in comparison with the lung. This may describe the lack of preventive effect of captopril on LPO in hepatocytes in our study.

In conclusion, pretreatment with captopril (a thiol ACE inhibitor) can prevent damage to hepatocytes induced by PQ and therefore, may be considered a therapeutic agent in the prevention and treatment of environmental toxins such as PQ. More studies are needed to elucidate the exact mechanism by which captopril protects against PQ toxicity.

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References
