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Enhancement of Cyclosporine-Induced Oxidative Damage of Kidney Mitochondria by Iron

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The present study investigated the stimulatory effects of iron (or ascorbate) on cyclosporine-induced kidney mitochondrial damage. Damaging effect of 50 μM cyclosporine plus 20 μM Fe^{2+} on mitochondrial lipids and proteins of rat kidney and hyaluronic acid was greater than the summation of oxidizing action of each compound alone, except sulfhydryl oxidation. Cyclosporine and 100 μM ascorbate showed an enhanced damaging effect on lipids but not on proteins. The peroxidative action of cyclosporine on lipids was enhanced with increasing concentrations of Fe^{2+}. Ferric ion (20 μM) also interacted with cyclosporine to stimulate lipid peroxidation. Damaging action of cyclosporine on mitochondrial lipids was enhanced by ascorbate (100 μM and 1 mM). Iron chelators, DTPA and EDTA, attenuated carbonyl formation induced by cyclosporine plus ascorbate. Cyclosporine (100 μM) and 50 μM Fe^{2+} (or 100 μM ascorbate) synergistically stimulated degradation of 2-α deoxyribose. Cyclosporine (1 to 100 μM) reduced ferric ion in a dose dependent manner, which is much less than ascorbate action. Addition of Fe^{2+} caused a change in absorbance spectrum of cyclosporine in 230~350 nm of wavelengths. The results show that cyclosporine plus iron (or ascorbate) exerts an enhanced damaging effect on kidney mitochondria. Iron and ascorbate appear to promote the nephrotoxicity induced by cyclosporine.

Key Words: Kidney mitochondria, Cyclosporine, Iron, Enhancement of oxidative tissue damage

INTRODUCTION

Cyclosporine has been used in the prevention and treatment of organ transplant rejection and in the treatment of autoimmune diseases (Kahan, 1989). The clinical uses of the drug are restricted by side effects, such as nephrotoxicity, hypertension, hepatotoxicity and neurotoxicity. The underlying mechanisms leading to nephrotoxicity are not clearly investigated. A number of mediators and mechanisms have been proposed as the cause of cyclosporine-induced nephrotoxicity (Baliga et al, 1997). The causative factors appear to include renal vasoconstriction, metabolism of the drug, thiol oxidation and formation of free radicals.

Role of free radicals has been postulated in the pathogenic mechanism of the cytotoxicity of cyclosporine (Ahmed et al, 1993; Durak et al, 1998). The potential sources of free radicals in the kidney include NADPH-oxidase mediated formation of oxidants in mesangial cells, uncoupling of cytochrome P450 monooxygenase system and mitochondrial respiratory chain. In cultured rat hepatocytes, cyclosporine increases reactive oxygen production and formation of thiobarbituric acid (TBA) reactive substances, induces loss of protein thiols and decreases the ratio of GSH/GSSG (Wolf et al, 1997). Reactive oxygen species are produced during metabolism of cyclosporine. Administration of cyclosporine into renal transplant patients (McGrath et al, 1997) and animals (Durak et al, 1998) caused alteration of endogenous antioxidant enzyme activities. Antioxidants, including vitamin E, inhibit hydrogen peroxide

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production induced by cyclosporine in cultured human mesangial cells (Perez de Lema et al, 1998), reduce lipid peroxidation of renal microsomes (Wang et al, 1995), prevent change of antioxidant enzyme activity in the kidney tissues and restore a decreased renal function.

Metal ions significantly catalyze formation of highly reactive oxygen species and effectively promote the oxidative tissue damage. The oxidoreduction of iron is considered to play a central role in lipid peroxidation (Halliwell & Gutteridge, 1989). The autoxidation of iron produces reactive oxygen metabolites, such as hydroxyl radical, perferryl ion and ferryl ion, and these oxygen metabolites appear to be involved in the initiation of lipid peroxidation. The kidney, particularly the proximal tubule, contains a large amount of ferritin (Fleming, 1987). Other sources of iron within the kidney are heme proteins including mitochondrial cytochromes, cytochrome P450, and catalase. Extrarenal heme proteins, such as hemoglobin and myoglobin, might also serve as sources of iron (Shah & Walker, 1988). During hypoxia/reoxygenation, release of iron from proximal tubule epithelial cells and hydroxyl radical formation is increased. Preincubation of the cells with cytochrome P450 inhibitors reduces iron release and hydroxyl radical formation during reoxygenation and prevent hypoxic renal cell injury (Paller & Jacob, 1994).

Erythrocytosis is prevalent in cyclosporine-treated renal transplant patients (Kessler et al, 1996). Nonenzymatic ascorbate-induced lipid peroxidation is increased in cyclosporin A-administered rats (Adhirai & Selvam, 1997). The iron chelator desferoxamine is reported to inhibit cyclosporine-induced contraction of renal cortex glomeruli, and implications of hydroxyl radical and lipid peroxidation in glomerular contraction are suggested (Wolf et al, 1994). Cytochrome P450 inhibitors attenuate cyclosporine-induced microsomal peroxidation by inhibition of metabolism (Serino et al, 1993). Thus, role of iron in cyclosporine-induced nephrotoxicity is suggested. However, direct effect of metal ions, including iron, on the cytotoxic action of cyclosporine and interaction of iron with cyclosporine have not been elucidated.

The present study evaluated the promoting action of iron on the cytotoxic action of cyclosporine on the kidney. The stimulatory effects of iron and ascorbate on the damaging action of cyclosporine on the mitochondrial lipids, proteins and sulfhydryl groups in the kidney cortex and hyaluronic acid, a mucopolysaccharide, were examined. We also explored by which mechanism they promote cyclosporine cytotoxicity. The results show that cyclosporine plus iron (or ascorbate) exerts an enhanced damaging effect on kidney mitochondria. Iron and ascorbate appear to promote the nephrotoxicity induced by cyclosporine.

METHODS

Materials

Cyclosporine (Sandimmun Neoral) was obtained from Sandoz Pharmaceutical Ltd., Switzerland. Superoxide dismutase (from bovine blood), catalase (from bovine liver), dimethyl sulfoxide, 1,4-diazabicyclo (2,2,2) octane, NADPH, NADH, glutathione (reduced form), mannitol, diethylenetriamine pentaacetic acid (DTPA), ethylenediamine-tetraacetic acid, ascorbic acid, 2-thiobarbituric acid, 2,4-dinitrophenylhydrazine, guanidine, hyaluronic acid (Grade III from human umbilical cord), 5,5'-dithiobis-(2-nitrobenzoic acid), glutathione reductase, 2-α deoxyribose and o-phenanthroline were purchased from Sigma-Aldrich Inc. (ST. Louis, MO USA). Other chemicals were of analytical grade. In Figures and Tables, cyclosporine is expressed as CsA.

Isolation of kidney cortex mitochondria

Mitochondria were prepared from the kidney cortex of male Sprague-Dawley rats weighing about 200 g according to the method of Mingatto et al (1996). The kidney cortex was removed and was sliced in a medium I (250 mM mannitol, 70 mM sucrose, 10 mM HEPES, 1 mM EDTA, 120 mM KCl and 50 mM Tris-HCl, pH 7.4). The tissue was homogenized with polytron homogenizer (Brinkman, model PT-20). The homogenate was centrifuged at 755 g for 5 min, and the resulting supernatant was centrifuged at 13,300 g for 15 min. The pellet was suspended in a medium II (250 mM mannitol, 70 mM sucrose, 10 mM HEPES and 50 mM Tris-HCl, pH 7.4, or 120 mM KCl and 50 mM Tris-HCl, pH 7.4) and was washed two times with the same buffer by centrifugation at 13,300 g for 15 min. The final mitochondrial pellet was suspended in a medium II. Mitochondrial protein was determined by the Bradford method using Bio-Rad protein assay kit.

Measurement of lipid peroxidation

Lipid peroxidation of mitochondria was estimated by measuring malondialdehyde (MDA) concentration using thiobarbituric acid (TBA) method. Mitochondria (0.4 mg protein/ml) were suspended in the reaction mixture consisting of 150 mM KCl, 0.1 mM sodium azide and 50 mM NaH_2PO_4, pH 7.4. Reaction was started by addition of oxidant, and the final volume was 1.0 ml. After 30 min of incubation, the reaction was stopped by adding 1.0 ml of 1% TBA in 50 mM NaOH and 1.0 ml of 2.8% trichloroacetic acid. The chromophore was developed by boiling, and the absorbance was measured at 532 nm (Gutteridge et al, 1982). The MDA concentration was expressed as nmol/mg protein using the molar extinction coefficient of 1.56×10^5/M/cm (Buege & Aust, 1978).

Measurement of carbonyl groups

The oxidation of mitochondrial proteins was measured by carbonyl assay using 2,4-dinitrophenylhydrazine (DNPH) (Levine et al, 1993). One ml of reaction mixtures, which contained 1 mg protein/ml of mitochondria, were treated with cyclosporine and Fe^{2+} (or ascorbate) for 30 min. Four ml of 10 mM DNPH in 2.5 M HCl was added to mixtures, and tubes were left for 1 h at room temperature in the dark. The mixtures were treated with 20% and 10% of trichloroacetic acid solution sequentially. After centrifugation, pellets were washed three times with 4 ml of ethanol : ethyl acetate (1 : 1) solution. The final pellets were dissolved in 2 ml of 6 M guanidine HCl solution and were left for 15 min at 37°C with mixing. Absorbances of the supernatants were read at 370 nm, and protein carbonyls were calculated using the molar extinction coefficient of 2.2×10^4/M/cm.

Measurement of hyaluronic acid degradation

Viscosity of hyaluronic acid was measured in a modified Cannon capillary viscometer that is regulated by vacuum pressure. The reaction mixtures contained 1 mg/ml hyaluronic acid, 150 mM KCl, 50 mM KH_2PO_4 buffer, pH 7.5 and other compounds. The viscosity change of depolymerized hyaluronic acid was measured at 25°C and expressed as a flow time (sec).

Measurement of GSH content

Mitochondria (1.0 mg protein/ml) were incubated in 100 mM Tris-HCl, pH 7.4 buffer medium and were treated with cyclosporine for 30 min at 37°C. The assay mixture (1.0 ml) was mixed with the same volume of 2 M $HClO_4$ and was centrifuged at 12,000 g for 8 min. The supernatant (100 μl) was added to assay mixture containing 0.6 mM DTNB, 200 μM NADPH and 0.1 M potassium phosphate, pH 7.0 to make a total volume of 980 μl. Twenty μl of GSH reductase (6 U/ml) was added to initiate the assay. The formation of 5-thio-2-nitrobenzoic acid was measured at 412 nm. The amount of total GSH was determined from a standard curve obtained with known amounts of GSH standards (Hung et al, 1998).

Measurement of thiobarbituric acid reactivity of 2-α deoxyribose

Amount of hydroxyl radical generated was estimated from TBA reactivity of 2-α deoxyribose (Aruoma, 1994). The reaction mixtures contained 2 mM 2-α deoxyribose, 100 μM cyclosporine, 50 μM $FeSO_4$, (or 100 μM ascorbate), 150 mM KCl and 50 mM NaH_2PO_4 buffer, pH 7.4. The reaction was performed for 30 min and was stopped by adding 1.0 ml of 1% TBA in 50 mM NaOH and 1.0 ml of 2.8% trichloroacetic acid. The reaction mixtures were heated in a boiling water bath, and then the absorbance was read at 532 nm.

Assay of reducing ability

Reducing action of cyclosporine on iron was assayed using o-phenanthroline (Aust et al, 1993). The assay mixture consisted of 100 μM $FeCl_3$, 10 mM o-phenanthroline, 150 mM KCl and 50 mM NaH_2PO_4, pH 7.4. Reaction was initiated by addition of either cyclosporine or ascorbate at 37°C, and absorbance change was traced at 510 nm.

Statistical analysis

Data were expressed as means $\pm$ S.D.. The results obtained in various experiments were analyzed for significant differences using the SAS program (SAS Institute Inc. USA). Nonparametric Wilcoxon rank sum test was applied on the comparison of differences between groups.

RESULTS

Promoting actions of iron and ascorbate on cyclosporine-induced oxidative damage

Cyclosporine cytotoxicity was evaluated with peroxidation of lipids and proteins of the kidney cortex mitochondria and degradation of carbohydrates using hyaluronic acid. Concentration of cyclosporine used in this study is based on previously reported experimental data (Ahmed et al, 1993; Serino et al, 1993; Wolf et al, 1997). Iron, which is presented in μM concentrations within the tissues, is well known to promote tissue damage caused by oxidants (Halliwell & Gutteridge, 1989). In this respect, influence of iron on cyclosporine-induced cytotoxicity was elucidated. Table 1 shows the enhanced damaging effect of cyclosporine and Fe^{2+} (or ascorbate) on tissue components. Cyclosporine (50 μM) caused damage of mitochondrial lipids and proteins, and for incubation of 30 min, 1.94 nmol/mg protein of MDA and 1.01 nmol/mg protein of carbonyls were formed. Damaging effect of cyclosporine plus Fe^{2+} (20 μM) on mitochondrial lipids and proteins was greater than summation of oxidizing actions of cyclosporine alone and Fe^{2+} alone.

The cellular physiological concentrations of ascorbate are reported to be in the range of μM to mM. Low concentrations of ascorbate appear to stimulate production of reactive oxidant, hydroxyl radical, and show pro-oxidant action (Halliwell & Gutteridge, 1989). Similar to cyclosporine and Fe^{2+}, the promoting effect was seen in the damaging actions of cyclosporine and ascorbate on mitochondrial lipids, while the effect was not observed in protein oxidation. Sulfhydryl groups of mitochondria were oxidized by addition of 50 μM cyclosporine, and at 30 min of incubation, 54.2% was oxidized. In contrast to the formation of MDA and carbonyls, cyclosporine and Fe^{2+} (or ascorbate) did not show an increased damaging effect on sulfhydryl groups. In contrast to lipids and proteins, damaging effect of 50 μM cyclosporine on hyaluronic acid was greater than that of 20 μM Fe^{2+}. Equal concentrations of cyclosporine and Fe^{2+} decreased viscosity of hyaluronic acid by 3.18 sec and 1.33 sec, respectively. Cyclosporine plus Fe^{2+} (or 100 μM ascorbate) also exhibited an enhanced damaging effect on hyaluronic acid.

In Fig. 1, the promoting action of iron on the cyclosporine-induced peroxidation of mitochondrial lipids is shown. The peroxidative action of cyclosporine was enhanced with increasing concentrations of Fe^{2+}. At 20 μM, Fe^{2+} in the presence of 50 μM cyclosporine formed 9.89 nmol/mg protein of MDA, while iron alone produced 4.04 nmol/mg protein of MDA, and thus exhibited a 65.4% of promoting effect. Equal concentration of ascorbate and 10 μM Fe^{2+} showed a 13.2% stimulation, and the addition of 50 μM Fe^{2+} revealed a plateau effect. Similar to catalyzing action of Fe^{2+}, ferric ion (20 μM) also interacted with cyclosporine and showed a 30.1% potentiation in lipid peroxidation. The stimulatory

Table 1. Effects of iron (II) and ascorbate on cyclosporine-induced oxidative damage

Compounds	MDA (n=4)	Carbonyls (n=4)	SH groups (n=5)	Hyaluronic acid (n=3)
No addition	–	–	22.46±0.72	–
CsA 50 μM	1.94±0.10	1.01±0.04	10.28±0.34	3.18±0.36
Fe^{2+} 20 μM	4.04±0.42	2.53±0.54	21.54±0.16	1.33±0.18
CsA 50 μM + Fe^{2+} 20 μM	9.89±0.38*	4.74±0.83*	9.53±0.15	5.23±0.28*
Ascorbate 100 μM	1.95±0.07	1.04±0.10	18.07±0.22	0
CsA 50 μM + Ascorbate 100 μM	5.56±0.15†	2.10±0.20	8.33±0.19	5.14±0.30†

Values in MDA, carbonyls and SH groups are nmol/mg protein. Values in hyaluronic acid represent viscosity (sec) decreased by compounds. Viscosity of intact hyaluronic acid was 19.1±0.4 sec. Data are means±S.D.. *$p<0.05$; significantly different from summation of CsA alone and Fe^{2+} alone, and $^{\dagger}p<0.05$; significantly different from CsA alone plus ascorbate alone.

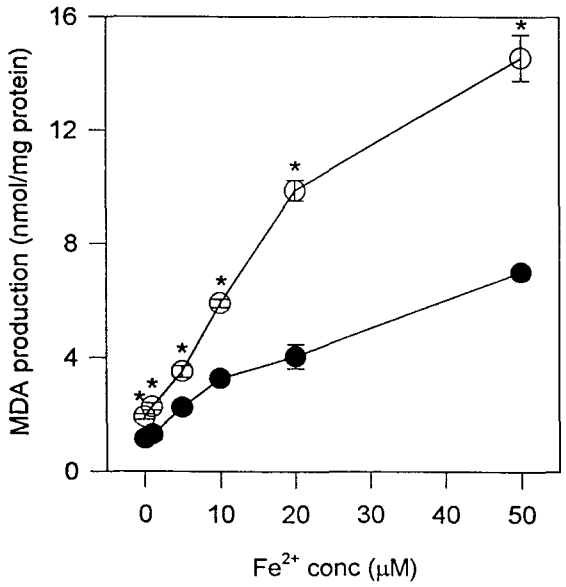

Fig. 1. Catalyzing action of iron (II) on cyclosporine-induced lipid peroxidation. Kidney mitochondria (0.4 mg protein/ml) were treated with varying concentrations of $FeSO_4$ in the presence (○) or absence (●) of 50 μM cyclosporine. Data are means±S.D., n=4. *$p<0.05$; significantly different from CsA alone plus Fe^{2+} alone.

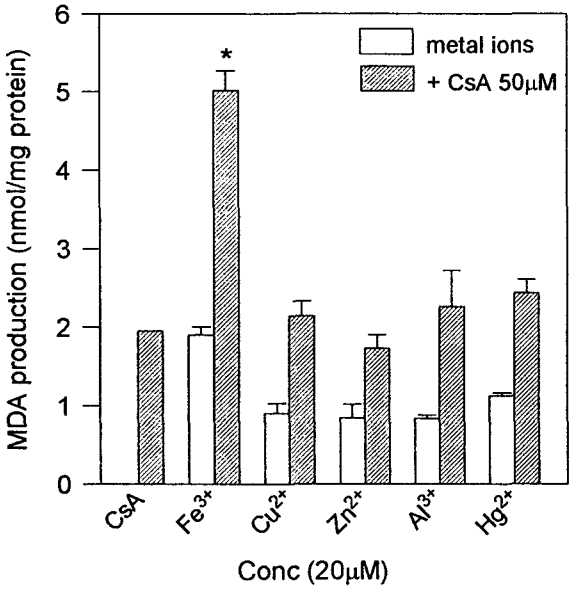

Fig. 2. Effects of metal ions on lipid peroxidation caused by cyclosporine. Kidney mitochondria were treated with various metal ions in the presence of 50 μM cyclosporine. Data are means±S.D., n=4. *$p<0.05$; significantly different from CsA alone plus Fe^{3+} alone.

Table 2. Effects of cyclosporine and ascorbate on peroxidation of mitochondrial lipids

Compounds	MDA production (nmol/mg protein)	+ CsA 50 μM
No addtion	1.16±0.03	2.11±0.12
Ascorbate 10 μM	1.19±0.05	2.13±0.06
Ascorbate 100 μM	1.95±0.07	5.76±0.18^{+}
+ DTPA 1 mM	–	1.05±0.10*
+ EDTA 1 mM	–	0.88±0.05*
Ascorbate 1 mM	1.10±0.17	6.66±0.17^{+}

Values are nmol/mg protein and are means±S.D., n=4. *$p<0.05$; significantly different from CsA 50 μM plus ascorbate 100 μM, and $^{+}p<0.05$; significantly different from ascorbate alone.

action of iron on cyclosporine effect was not detected in other metal ions as can be seen in Fig. 2. At equal concentration with iron, Cu^{2+}, Zn^{2+}, Al^{3+} and Hg^{2+} (all 20 μM) did not promote the peroxidative action of cyclosporine on mitochondrial lipids and revealed additive effects.

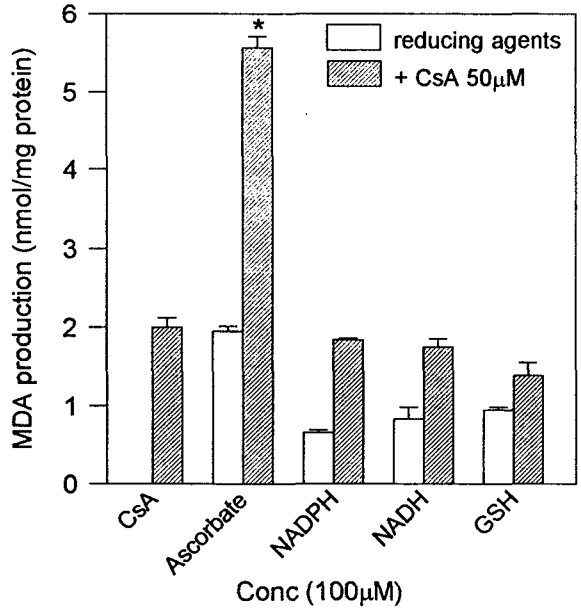

Fig. 3. Enhancement of cyclosporine-induced peroxidation of mitochondrial lipids by ascorbate. Kidney mitochondria were treated with 50 μM cyclosporine and reducing agents. Data are means±S.D., n=4. *$p<0.05$; significantly different from CsA alone plus ascorbate alone.

Role of iron reducing action in the cyclosporine-induced lipid peroxidation was explored. Fig. 3 and Table 2 show that the damaging action of cyclosporine on mitochondrial lipids was enhanced by ascorbate (100 μM and 1 mM) in a synergistic manner. One mM ascorbate in the presence of cyclosporine greatly increased MDA formation, while the same concentration of ascorbate alone did not elicit peroxidation of lipids. DTPA, a specific iron chelator, and EDTA (1 mM) markedly attenuated the peroxidative action of cyclosporine plus ascorbate. On the other hand, the stimulatory effects of NADPH, NADH and GSH (all 100 μM) on cyclosporine action were not observed. In contrast to same concentration of ascorbate, they did not cause peroxidation of lipids, and particularly GSH rather exhibited a protective effect.

Table 3 represents the inhibition of cyclosporine plus Fe^{2+} (or ascorbate)-induced oxidation of mitochondrial lipids and proteins by oxidant scavengers and metal chelators. Concentrations of oxidant scavengers used in the present study are based on reported data (Serino et al, 1993; Sugiyama et al, 1996; Koh et al, 1998), and they themselves did not show any damaging effects on mitochondria (data not shown). Cyclosporine plus Fe^{2+}-evoked peroxidation of mitochondrial lipids was decreased by addition of 30 μg/ml SOD, a scavenger of superoxide anion, and 30 μg/ml catalase, a scavenger of H_2O_2, but was not affected by 10 mM DMSO, a scavenger of hydroxyl radical, and 10 mM DABCO, a quencher of singlet oxygen. Equal concentrations of DMSO and DABCO attenuated the carbonyl formation caused by Fe^{2+} and cyclosporine, whereas 30 μg/ml of SOD and catalase did not show any significant effects. The damaging action of cyclosporine plus ascorbate on proteins was almost completely depressed by 1 mM of DTPA and EDTA.

Table 3. Effects of oxidant scavengers and iron chelators on MDA and carbonyl formation caused by cyclosporine

Compounds	MDA (nmol/mg protein)	Carbonyls (nmol/mg protein)
CsA 50 μM	1.94±1.10	1.01±0.05
Fe^{2+} 20 μM	4.04±0.42	2.53±0.54
CsA 50 μM + Fe^{2+} 20 μM	9.89±0.29	4.74±0.83
+ SOD 30 μg/ml	7.55±0.10*	4.74±0.95
+ Catalase 30 μg/ml	7.95±0.35*	3.94±0.65
+ DMSO 10 mM	9.44±0.15	3.21±0.74*
+ DABCO 10 mM	9.03±1.11	2.78±0.66*
Ascorbate 100 μM		1.04±0.10
CsA 50 μM + Ascorbate 100 μM		2.10±0.20
+ DTPA 1 mM		1.07±0.15†
+ EDTA 1 mM		1.01±0.08†

Data are means±S.D., n=4. *$p<0.05$; significantly different from 50 μM CsA plus 20 μM Fe^{2+}, and †$p<0.05$; significantly different from 50 μM CsA plus 100 μM ascorbate.

Effects of cyclosporine and iron(II) on deoxyribose degradation and iron reduction

Hydroxyl radical production caused by interaction of cyclosporine with Fe^{2+} (or ascorbate) was assayed using 2-α deoxyribose degradation. As shown in Fig. 4, Fe^{2+} (50 μM) plus 100 μM cyclosporine synergistically stimulated degradation of 2-α deoxyribose and showed a 49.5% potentiation, which was significantly inhibited by 10 mM of DMSO and man-

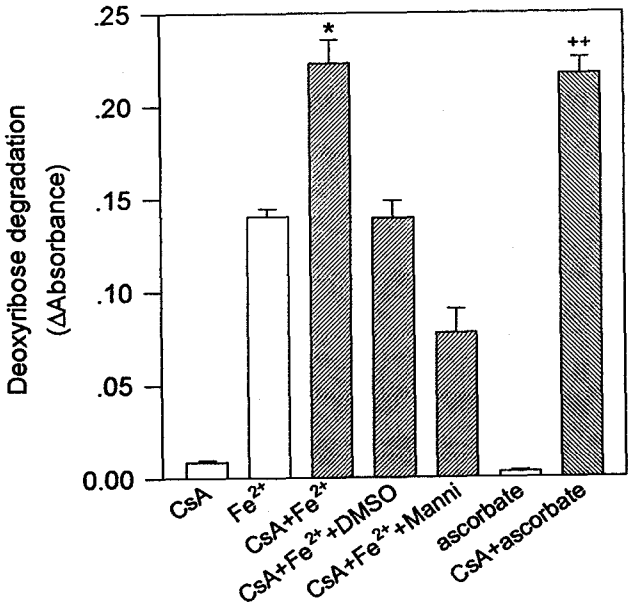

Fig. 4. Cyclosporine and iron-induced deoxyribose degradation. Cyclosporine (100 μM) and 50 μM $FeSO_4$ (or 100 μM ascorbate) were added to reaction mixtures containing 2 mM of 2-α deoxyribose. DMSO, 10 mM DMSO and Manni, 10 mM mannitol. *$p<0.05$ and ++$p<0.01$; significantly different from CsA alone + Fe^{2+} alone and CsA alone+ascorbate alone, respectively.

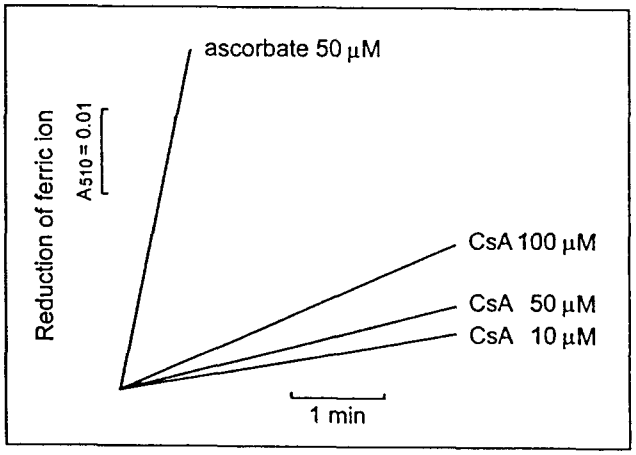

Fig. 5. Reduction of ferric ion by cyclosporine. Varying concentrations of cyclosporine were added to reaction mixtures containing $FeCl_3$ (100 μM) and o-phenanthroline.

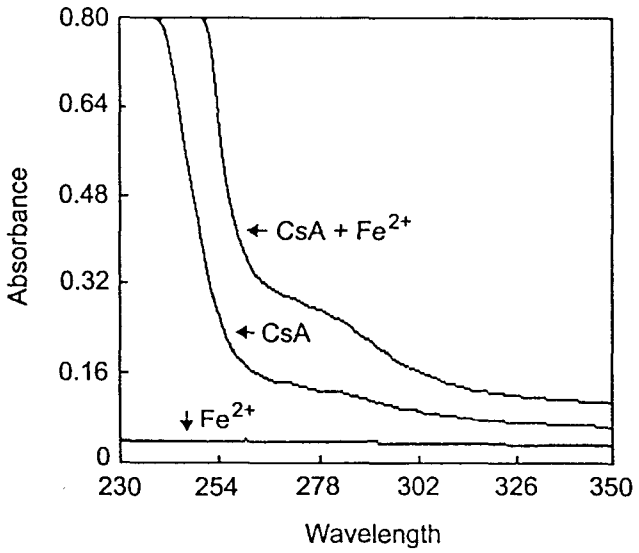

Fig. 6. Change of cyclosporine absorbance spectrum by addition of iron (II). Cyclosporine (50 μM) was mixed with 20 μM $FeSO_4$.

nitol. Similar finding was also investigated in reaction mixture containing cyclosporine and ascorbate. Ascorbate (100 μM) alone did not show a damaging effect on 2- α deoxyribose. However, ascorbate in the presence of cyclosporine caused an about 24.4 times of enhancement compared to additive effect of each compound.

The reducing action of cyclosporine on ferric ion is represented in Fig. 5. Cyclosporine (1 to 100 μM) reduced ferric ion in a dose dependent manner. On a concentration base, the reducing ability of cyclosporine was much weaker than ascorbate.

In Fig. 6, the addition of 20 μM Fe^{2+} caused a change in absorbance spectrum of cyclosporine (50 μM) in wavelength range of 230~350 nm. In this wavelength range, unlike cyclosporine, Fe^{2+} alone did not have an absorbance.

DISCUSSION

Iron catalyzes effectively tissue damage caused by oxidants. Autoxidation of iron produces reactive oxygen metabolites which cause the damage of biological molecules. Catalytic iron is released from mitochondrial cytochrome P450 during hypoxia/reperfusion (Paller & Jacob, 1994) and from mitochondria exposed to the nephrotoxin gentamicin (Baliga et al, 1997). It is suggested that cytochrome P450 is the critical source of iron, and additional iron may be released after cells are irreversibly damaged. Cytochrome P450 inhibitors are demonstrated to attenuate hydroxyl radical production induced by reoxygenation and to inhibit cyclosporine-caused peroxidation of liver microsomes (Serino et al, 1993). Desferoxamine, an iron chelator, attenuates contraction of renal cortex glomeruli caused by cyclosporine. Despite catalyzing action of iron in gentamicin-induced nephrotoxicity and in reperfusion-evoked renal injury, the role of iron in cyclosporine nephrotoxicity has not been elucidated.

Stated concentrations of cyclosporine and iron (II or III), respectively, caused the oxidative damage of lipids, proteins and sulfhydryl groups of kidney mitochondria and hyaluronic acid, a mucopolysaccharide. The result indicates that administered cyclosporine may express a cytotoxic action on all tissue components, lipids, proteins and carbohydrates, in the body. Cyclosporine plus iron exhibited a synergistic damaging effect on tissue components tested except sulfhydryl groups, and the effect was greater than additive effect of cyclosporine alone and iron alone. Similar to the action of cyclosporine plus iron, cyclosporine in the presence of ascorbate revealed an enhanced damaging effect on mitochondrial lipids and hyaluronic acid, whereas this effect was not observed in protein components, carbonyls and sulfhydryl groups. Data represent that the interaction of cyclosporine with iron or ascorbate appears to promote the cyclosporine-induced destruction of renal

cells.

Peroxidation of cellular membrane lipids is associated with alteration of membrane function and inactivation of integral enzymes. Catalyzing action of Fe^{2+} on cyclosporine effect was prominent in peroxidation of mitochondrial lipids. Enhanced damaging effect due to interaction of cyclosporine with iron could be supported by enhancement of the cyclosporine-induced peroxidation of lipids with increasing concentrations of Fe^{2+}. Iron-oxygen complexes are considered as causative agents in lipid peroxidation rather than reactive oxidant, hydroxyl radical (Halliwell & Gutteridge, 1989). Formation of cyclosporine-iron complexes might be postulated by no inhibitory effect of DMSO on peroxidative action of cyclosporine plus Fe^{2+} on lipids and by change of absorbance spectrum of cyclosporine treated with Fe^{2+}. We also observed that in the presence of fixed concentration of Fe^{2+}, lipid peroxidations of the kidney mitochondria and microsomes were enhanced with increasing concentrations of cyclosporine (data not shown). This finding also supports a possible formation of cyclosporine-iron complex.

A number of transition metal ions act as effective catalysts for the peroxidation of unsaturated fatty acids. In this study, effects of various metal ions on cyclosporine-induced lipid peroxidation were examined and were compared to that of iron. Similar to Fe^{2+}, Fe^{3+} (20 μM) in the presence of cyclosporine also showed an enhanced damaging action on mitochondrial lipids, but the promoting effect of Fe^{3+} was less than that of Fe^{2+}. The stimulatory effect of Fe^{3+} on cyclosporine action may be ascribed partially to iron reducing ability of cyclosporine. The metal ion-catalyzing effect on lipid peroxidation in medium containing cyclosporine was not seen in other metal ions, such as Cu^{2+}, Zn^{2+}, Al^{3+} and Hg^{2+}. These findings suggest that redox state of iron affects cyclosporine cytotoxicity, and iron has a selective affinity to cyclosporine.

The reducing agents, including ascorbate and NADPH, interact with metal ions to promote formation of reactive oxidants and stimulate oxidative tissue damage (Halliwell & Gutteridge, 1989). The present data exhibited differential effects of reducing agents on the cyclosporine-induced peroxidation of mitochondrial lipids. Ascorbate is known to exhibit antioxidant ability or prooxidant action depends on its concentration. Administration of ascorbate improves the activities of antioxidant enzymes, such as SOD and glutathione peroxidase, and partially attenuates subcellular damage. The cytotoxic action of cyclosporin A on cultured rat hepatocytes is attenuated by addition of ascorbate (Wolf et al, 1997). However, ascorbate is reported to enhance dopamine-induced apoptosis of PC12 cells (Si et al, 1998). The ascorbate-induced lipid peroxidations of the kidney and liver are enhanced in cyclosporine-treated rats (Adhirai & Selvam, 1997). In the present experiment, ascorbate enhanced lipid peroxidation evoked by cyclosporine in a dose dependent manner up to 1 mM, while 1 mM ascorbate alone did not cause lipid peroxidation. The protective effects of iron chelators, DTPA and EDTA, on lipids and proteins postulate involvement of iron in the peroxidative action of cyclosporine plus ascorbate.

In contrast to ascorbate, NADPH, NADH and GSH did not stimulate the peroxidative action of cyclosporine on lipids but rather slightly inhibited it. At 100 μM of concentration, they themselves did not show peroxidative actions. This finding suggests a possible specific interaction of ascorbate with cyclosporine. Compared to enzymatic lipid peroxidation by Fe^{3+}, ADP and NADPH (Halliwell & Gutteridge, 1989), stimulating effect of NADPH or NADH on cyclosporine-induced peroxidation are not suggested. Reducing action of NAD(P)H on and their interactions with cyclosporine may not play significant roles in the cytotoxicity. GSH detoxify free radicals formed during intermediary metabolism and drug detoxication. Cyclosporine inhibits GSH S-transferase, which is probably associated with its cytotoxicity (Hoffman et al, 1995). The weakening or enforcement of the cellular glutathione state is known to increase or decrease cyclosporine cytotoxicity (Walker et al, 1990; Zhang & Lindup, 1996; Wolf et al, 1997). Coincided with these reports, exogenous GSH showed a protective action on cyclosporine-induced lipid peroxidation.

Involvement of oxygen free radicals in the damages of mitochondrial lipids and proteins induced by cyclosporine and Fe^{2+} could be inferred from protective effects of antioxidants. The peroxidations of mitochondrial lipids and proteins caused by cyclosporine plus Fe^{2+} differently responded to oxidant scavengers. DMSO and DABCO significantly decreased the cyclosporine plus Fe^{2+}-induced carbonyl formation but not affected lipid peroxidation. In the same experimental conditions, inhibitory effects of SOD and catalase on carbonyl formation were not

seen different from lipid peroxidation. Thus, cell components may differently respond to oxidants in the cyclosporine-induced damage of the kidney. TBA reactivity of 2-α deoxyribose is used as a sensitive detection method for hydroxyl radical and reflects damage of monosaccharides (Griffiths & Lunec, 1996). Hydroxyl radical scavengers, DMSO and mannitol significantly inhibited the degradative action of cyclosporine plus Fe^{2+} on deoxyribose. The stimulatory effect of cyclosporine plus Fe^{2+} (or ascorbate) on deoxyribose degradation indicates that their interactions produce effectively hydroxyl radical and similar to hyaluronic acid, cause enhanced damaging effects on carbohydrates.

Reducing ability of cyclosporine on iron was explored using o-phenanthroline. Cyclosporine showed a reducing action on $FeCl_3$, which sensitively responded to ascorbate. Although the action of cyclosporine at the concentration base was very weaker than that of ascorbate, the reducing ability of cyclosporine on iron could be supported by its dose dependent effect, and this ability may affect the iron-catalyzing cyclosporine cytotoxicity. In conclusion, cyclosporine plus iron (or ascorbate) shows an enhanced damaging effect on kidney mitochondria. Iron and ascorbate appear to promote cyclosporine-induced nephrotoxicity.

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