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Comparison of cyclosporine and FK506 effects on glutathione levels in rat cochlea, brain, liver and kidney

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Abstract—Cyclosporine treatment (50 mg/kg/day, p.o.) caused increases in rat renal reduced glutathione (GSH) levels of 205 and 673%, respectively, after 5 and 10 days. No changes were seen in liver GSH with either dose of cyclosporine. FK506 (2.5 mg/kg/day, p.o., for 7 days) caused an approximately 200% increase in kidney GSH, and an approximately 250% increase in hepatic GSH levels. Oxidized glutathione (GSSG) was never more than 1–2% of the level of the reduced form in any tissue from control animals. Small increases in the ratios of oxidized to reduced glutathione were seen in livers and kidneys from both cyclosporine- and FK506-treated animals. No changes in GSH or GSSG levels were seen in brains or cochleas from any animal.

Cyclosporine is a cyclic polypeptide that is a potent immunosuppressive agent used in conjunction with transplant surgery and in the treatment of some autoimmune diseases. Its use can result in hepato- and nephrotoxicity [1]. FK506 is a macrolide antibiotic that is undergoing clinical trials as a new immunosuppressant. FK506 has been found to be hepatotoxic in primates [2] and dogs [3, 4]. Other organs adversely affected in dogs treated with FK506 include the heart and pancreas [4] and the vascular system [5].

Glutathione (GSH*) is a tripeptide which plays a major role in the detoxification of electrophilic drugs and metabolites and active oxygen formed during drug metabolism. GSH may participate in drug metabolism via reactions catalyzed by GSH *S*-transferases, transpeptidases, transhydrogenases, peroxidases and reductases. Inhibition or inactivation of this important detoxification mechanism may be the central factor in the potentiation of the toxicities of the aminoglycosides and loop diuretics, and may also be involved in the toxicities of ionizing radiation therapy, cisplatin and other agents.

Administration of cyclosporine has been reported to have variable effects on the concentration of GSH in different tissues [6, 7]. The effects of administration of FK506 on tissue GSH levels have not been reported previously. The present study is part of a project investigating toxic mechanisms of drugs causing oto- and nephrotoxicity, hepatotoxicity and neuropathies. We have compared drug effects on GSH levels in kidney and liver and in brain and cochlea.

Methods

Oxidized and reduced glutathione measurements. Both oxidized glutathione (GSSG) and reduced GSH levels were determined using HPLC with electrochemical detection, as described by Allison and Shoup [8]. The working electrode for the HPLC was prepared by placing triple-distilled mercury on a highly-polished dual gold electrode. Oxidized and reduced GSH were separated on an octadecylsilane column, using a mobile phase of 3% methanol:97% monochloroacetate, pH 3.0, at a flow rate of 1.0 mL/min. The electrodes were set at potentials of −1.0 V upstream and +0.15 V downstream. The upstream electrode reduces GSSG that has been separated from reduced GSH on the column, and thus permits its separate detection and quantification in a single chromatographic run. This detection is accomplished by oxidation at the downstream electrode. Sensitivity of this method is 3.5 pmol/sample for GSH and 5.7 pmol/sample for GSSG.

Tissue preparation. Male Wistar rats, approximately 300 g, were decapitated after ketamine anesthesia, and organs were minced and sonicated in HPLC running buffer containing disodium EDTA and mercaptosuccinic acid (internal standard). Cochleas were trimmed away from the temporal bone and sonicated. This rapidly (<15 sec) dissolves all soft tissue, leaving only the bony modiolus. Livers were perfused with, phosphate-buffered saline prior to homogenization. The supernatant was collected after centrifugation at 1700 *g*, and injected directly into the HPLC. Data which are shown in Fig. 1 are normalized per mg wet weight of the tissue (kidney, liver and brain), or per whole cochlea. Experimental results from drug administrations were compared, using Student's *t*-test, to the corresponding data from animals receiving the vehicle for that drug only.

Drug dosages and administration. All drugs and drug vehicles were given by gavage. Cyclosporine A (Sandoz Pharmaceuticals) was suspended in 10% ethanol in olive oil, and a dose of 50 mg/kg/day was administered for either 5 or 10 days. FK506 (Fugisawa Co., Ltd.) was suspended in 25% carboxymethylcellulose in water, and a dose of 2.5 mg/kg/day was given for 7 days. Control animals received either 10% ethanol in olive oil or 25% carboxymethylcellulose in water. Animals were killed for

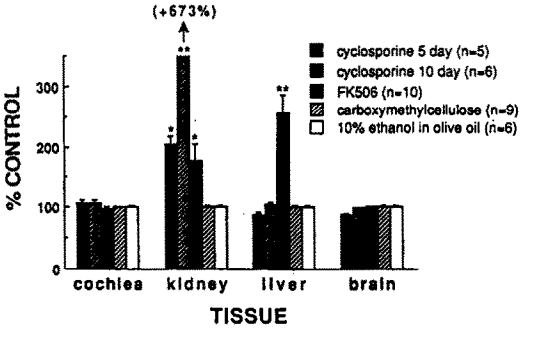

Fig. 1. Effects of cyclosporine and FK506 on glutathione levels *in vivo*. Doses and treatments were described in the text. Values are means ± SEM. Control values (10% olive oil in ethanol controls; mean ± SEM) were: cochlea, 15.3 ± 0.1 nmol/cochlea; kidney, 1649.0 ± 2.8 nmol/g wet wt; liver, 5104.1 ± 37.3 nmol/g wet wt; brain, 2007.9 ± 0.1 nmol/g wet wt. The carboxymethylcellulose control group means were within 10% of these values. Key: (*) $P < 0.01$, relative to control treatment; and (**) $P < 0.001$, relative to control treatment.

* Abbreviations: GSH, glutathione; and GSSG, oxidized glutathione.

the determination of GSH levels 24 hr after the last dose of drug or vehicle. Experimental results from animals receiving drugs or vehicles were compared to values obtained from untreated animals.

Results and Discussion

Neither drug vehicle affected GSH levels in any organ assayed, nor were brain and cochlea GSH levels altered by either cyclosporine or FK506 (Fig. 1). Kidney GSH levels were increased by 205 and 673%, respectively, after 5 and 10 days of treatment with cyclosporine (50 mg/kg/day). No changes were seen in the liver with either dose of cyclosporine. FK506 caused a 177% increase in kidney GSH, and a 256% increase in hepatic GSH levels. GSSG was never more than 1–2% of the level of the reduced form in any tissue from control animals. In cyclosporine-treated animals the percentage of GSH in the oxidized form in liver and kidney was increased slightly (to 3 and 5% of GSH levels, respectively). This small increase in the ratio of oxidized:reduced GSH was also found in kidneys of FK506-treated animals, while in the livers the percentage of the oxidized form was further increased to 8% of reduced GSH levels (GSSG data not shown). Such a change in the ratio of oxidized and reduced GSH in kidney after cyclosporine is also reported by Walker *et al.* [9].

Cyclosporine has been shown to stimulate lipid peroxidation in liver cells [10] and in renal cells and tissues [9, 11]. Free radical scavengers and antioxidants, including vitamin E, GSH and *N*-acetylcysteine, are reported to prevent this cyclosporine-induced lipid peroxidation [10]. There is also evidence that binding of cyclosporine to macromolecules, a likely step in toxicity, is a consequence of its metabolism by microsomal cytochrome P450, and that this binding is inhibited by GSH [12]. Cyclosporine has also been reported to inhibit several enzymes involved in the renal and hepatic mixed-function oxidases in several species of rats [13, 14]. FK506 inhibits its own metabolism by inhibition of hepatic cytochrome P450 mixed-function oxidase, which may lead to drug accumulation and toxicity [15].

Cyclosporine does not penetrate the blood–brain barrier to a significant extent [16], nor should FK506, based on its structure, and so it is unlikely that appreciable concentrations of either drug occur in the inner ear. Cyclosporine is known to be nephro- and hepatotoxic, but not to adversely affect hearing [17]. It is therefore not surprising that no effects were seen on cochlear GSH levels, although changes in cochlear GSH have been proposed as a factor in the ototoxicity of other agents [18, 19]. No changes were seen with either drug in brain [GSH], although both cyclosporine and FK506 are associated with apparent CNS toxicity. It is possible that this CNS toxicity is consequent to the peripheral damage in liver and kidney function which these drugs are known to cause, or reflects a direct toxic effect on the nervous system.

Conflicting results have been reported about the effects of cyclosporine on the GSH content of liver and kidney. Our results indicate profound increases in kidney GSH levels at both doses of cyclosporine, and no changes in other organs tested. These increases in kidney GSH are similar to those reported by Mayer and Cockett [7], who used doses in the same range as those in the present study. Our findings differ from those of Duruibe *et al.* [6] who found a significant reduction in GSH content of livers and kidneys taken from rats treated with cyclosporine. However, in this latter study, a much lower dose of cyclosporine (120 μg/kg/day) was given intraperitoneally. This difference in dose and route of administration, as well as the assay procedure, may be responsible for the differences in response of GSH in these tissues from what we observed.

Changes in tissue [GSH] may reflect both acute and subacute organ responses to toxic stress after drug treatment. There appears to be a temporal sequence of depletion and then a reactive stimulation of GSH synthesis. Acute depletion of GSH can result from its use in detoxification mechanisms, and can be a risk factor or indicator for potential toxicity. However, the subacute response to drug-induced toxic stress seems often to be an increased level of GSH. The large increases in some tissue GSH levels with cyclosporine or FK506 treatment may then be the subacute responses to the organ-specific toxicities reported with these drugs [1, 2, 4]. Such increases may represent an attempt by the tissue under toxic stress to enhance its capacity to inactivate reactive metabolites or active oxygen species. Alternatively, this may result from proliferation or selection of cells in the tissue with high GSH content. Certain drugs (e.g. cisplatin) may also inhibit enzymes which utilize GSH in detoxification [20]. This can also lead to increased GSH accumulation. Thus, the GSH system in kidney and liver treated with cyclosporine or FK506 may detoxify these drugs in these organs, as well as react to the toxic insult.

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Aging and the response of the isolated perfused rat liver to vasoactive drugs

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The clearance of many drugs is dependent on liver blood flow. Therefore, an age-related reduction in liver blood flow has important clinical implications for the elderly patient. Investigations using Indocyanine green clearance have shown that there is a senescence-related decline in liver blood flow of 49–53% in man [1, 2] and 35% in the rat [3]. It has been suggested that this is due mainly to reduced portal venous blood flow [4, 5], for which there are two possible causes: reduced splanchnic outflow or increased hepatic resistance to portal flow. The isolated perfused rat liver has been widely used to study hepatic resistance and its response to vasoactive substances, however little attention has been given to the effects of aging. In this study, we have measured the hepatic resistance in young and aged rats and its modulation by norepinephrine and verapamil. The action of verapamil was studied because it increases hepatic blood flow *in vivo* [6], antagonizes the norepinephrine-induced contractions of the isolated rat portal vein [7], and is used frequently in elderly patients.

Materials and Methods

Female Fischer F344 rats were obtained from Flinders Medical Centre (Adelaide, South Australia).

Liver perfusion. Rats were anaesthetized with pentobarbital (50 mg/kg i.p., Boehringer Ingelheim, Australia) and heparinized (200 U i.v., David Bull Laboratories, Australia). At laparotomy, the portal vein was cannulated with a 16G intravenous catheter and the liver was perfused with albumin-free Krebs–Henseleit buffer (pH 7.4, 1.19 mM $CaCl_2$, equilibrated with 95% O_2/5% CO_2) using a peristaltic pump (Cole-Parmer, IL, U.S.A.) in a non-recirculating system. The bile duct was cannulated with PE10 (Clay Adams, NJ, U.S.A.) and the liver isolated and maintained at 37° within a modified humidicrib. The vena cava was transected flush with the liver so that an outflow cannula was not necessary. Viability was assessed by bile flow, oxygen consumption and macroscopic appearance.

Measurement of portal pressure. A blunted 22G spinal needle was threaded through the portal vein cannula until the tip protruded 3–4 mm into the portal vein. Pressure was taken as the height of the column of perfusate in a vertical tube attached to the needle. Because the zero point was taken as the height of the vena cava outflow orifice, the transhepatic pressure gradient was measured directly. Correction was made for capillary action which was measured separately.

Perfusion protocol. Each liver was perfused with buffer initially and then, in order, with: buffer containing 10 μM norepinephrine (Winthrop, Australia); 10 μM norepinephrine and 20 μM verapamil (Knoll, Australia); 20 μM verapamil; and finally buffer alone to confirm that the pressures had returned to baseline. Pressure readings were taken at flow rates of 5, 10, 20 and 30 mL/min for each perfusate. At the completion of the whole experiment, the inflow line was clamped and the pressure recorded 3 min after cessation of flow. Hepatic resistance was calculated from the transhepatic pressure gradient divided by the flow rate.

Statistical analysis. The Student's *t*-test was used to