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Glycine attenuates Fanconi syndrome induced by maleate or ifosfamide in rats

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Glycine attenuates Fanconi syndrome induced by maleate or ifosfamide in rats. It has become widely recognized that glycine (Gly) depletion predisposes isolated proximal tubules (PT) to necrotic cell damage induced by diverse insults and that Gly replacement *in vitro* is highly cytoprotective. However, the effectiveness of supplementation with Gly *in vivo*, where blood and tissue Gly normally are maintained at high levels, is incompletely defined. Our aim was to assess whether: (a) supplementation of Gly in drinking water of rats would attenuate the proximal tubule damage and the Fanconi syndrome (FS) induced by maleate (Mal), a classical proximal tubule toxin, or ifosfamide (IFO), an antineoplastic drug; and (b) to explore the mechanisms responsible for such effects, since Gly supplementation might be especially beneficial in treating the FS, where the kidney tends to waste amino acids. Rats received daily injection of Mal (2 mmol/kg) for two days without or with oral supplementation of 2% Gly. IFO, 50 mg/kg, was injected daily for five days without or with oral Gly. Control rats were injected with saline, without or with oral Gly. The results demonstrated that both Mal and IFO induced a FS characterized by wasting of amino and organic acids, glucose, and electrolytes, along with elevated plasma creatinine (Crn) and BUN, and decreased Crn clearance rate. Light microscopy revealed a necrotic lesion in the proximal tubules of the Mal group, but no necrosis after IFO. Gly strongly ameliorated the severity of renal necrosis and/or dysfunction induced by Mal or IFO, with significant decreases in total and fractional excretion of Na^+, K^+, PO_4^{3-} and glucose, decreased plasma BUN and Crn, and increased Crn clearance. Analysis of freeze-clamped cortical tissue showed substantial depletion of [Gly], [ATP] and [GSH] along with increased GSSG in Mal or IFO groups and correction of [Gly] and [ATP] with Gly supplementation, but no improvement with Gly of reduced gluthatione [GSH] or the ratio of reduced to oxidized gluthatione (GSH/GSSG). ^{31}P-NMR analysis of the renal cortex indicated a decrease in Pi and various membrane phospholipids in Mal and IFO rats and prevention of this damage with Gly. These observations demonstrate that oral supplementation of Gly can provide protection against Mal or IFO-induced renal tubular cell dysfunction and structural damage. The lack of effect on glutathione oxidation and depletion suggests an action distal to toxin uptake and intracellular interactions, which is similar to the characteristics of Gly cytoprotection against diverse insults *in vitro*. The results also suggest modification by Gly of the primary toxicity of the agents and effects on phospholipid synthesis that could contribute to repair.

It has become widely recognized that glycine (Gly) depletion predisposes isolated proximal tubules to necrotic cell damage secondary to diverse insults and that restoration of Gly confers remarkable cytoprotection [1–6]. The mechanism for this effect, which is expressed in multiple cell types [7–13], remains incompletely defined, but, in its most essential form, involves suppression of a pathological plasma membrane permeability defect [14]. It has been unclear whether Gly supplementation *in vivo* in other than the transplant setting [15–20], is beneficial. Tissue Gly levels *in vivo* are normally well above those required for maximal cytoprotection, and the lack of efficacy of Gly supplementation during clamp models of ischemia reperfusion [16, 17] is likely accounted for by the continued presence of sufficient endogenous Gly during both the ischemic and reperfusion phases of the insult [21]. Heyman et al [15] have shown that parenteral Gly strongly protects against cisplatin-induced nephrotoxicity in rats, but this effect is associated with decreased renal uptake of the toxin [22]. In the present work, we wished to further assess the possibility and mechanisms for *in vivo* cytoprotection by Gly supplementation by an alternate route, that is, orally, during drug-induced renal dysfunction, and reasoned that such effects might be particularly relevant for lesions prominently characterized by Fanconi syndrome (FS), in which impaired amino acid absorption could cause depletion of Gly and alanine, another naturally abundant amino acid with similar cytoprotective effects [23]. Glycine has previously been shown to be cytoprotective in the cystine loaded isolated tubule model of FS [24].

Renal FS is characterized by a generalized disorder in proximal tubule (PT) transport and is associated with a number of disease states ranging from inherited disorders of metabolism to injury resulting from exposure to exogenous toxins [25–28], including chemotherapy treatment with ifosfamide (IFO) [29–34]. The use of IFO to treat malignant tumors in both children and adults very often has been associated with renal complications, including clinical and subclinical tubular nephrotoxicity and renal failure [30, 32, 34]. IFO is an alkylating antineoplastic drug that is chemically related to cyclophosphamide [30, 35], is active against many solid tumors, and in some cases has been found to be superior to cyclophosphamide [30, 35, 36]. IFO-induced nephrotoxicity is a major hazard of treatment [29–35] and limits the dose that can be administered [30, 35]. IFO may lead to a persistent Fanconi-syndrome and significant proximal renal tubular dysfunction associated with reduced GFR, glucosuria, aminoaciduria, phosphaturia and bicarbonaturia with proximal renal tubular acidosis [30, 32]. A widely studied animal model of the Fanconi

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syndrome associated with severe proximal tubule damage can be induced in dogs [37–39] and rats [40–45] by administration of maleate (Mal).

In the current work we have examined whether supplementation of Gly in the drinking water of rats can attentuate Mal or IFO-induced nephrotoxicity. The data demonstrate that Gly provides strong protection against the Mal or IFO-induced FS and proximal tubule damage in rats, an effect which is likely mediated by several beneficial actions.

Methods

Experimental procedure

Experiments were carried out on male Sprague-Dawley rats (Charles River) weighing 250 to 300 g and fed Purina rat chow and water *ad libitum*.

Our aim was to test the effect of glycine in two different models of experimental Fanconi syndrome *in vivo* at time points appropriate for expression of the lesion in each of the models. To that end, the design included control and four experimental groups as follows:

(1) Group Mal: Mal-induced renal injury. Rats maintained on Purina rat chow and water *ad libitum* were treated with intraperitoneal (i.p.) administration of two doses of 2 mmol/kg Mal. From a stock solution of 160 mg/ml of the disodium salt of Mal (Sigma# M9009), 0.5 ml was injected daily for two days, at the start of the experiment (time 0) and at 24 hours. Rats were housed individually in metabolic cages. Urine was collected to measure clearance and metabolite excretion between 8 and 24 hours after the second Mal injection (16 hr before rats were sacrificed). At approximately 24 hours following the second injection of Mal, rats were anesthesized with an i.p. injection of 50 mg/kg Na-Nembutal. The abdominal aorta was exposed and a 19 g needle with tubing was inserted into the vessel. Blood samples were obtained from the abdominal aorta and collected into heparinized tubes. In a separate series of experiments, kidneys were perfused *in situ* with a solution containing 100 mM Na-cacodylate and 2% glutaraldehyde for morphologic studies, as previously described [46]. In addition, a parallel series of experiments was carried out for determination of cortical metabolites. To this end, a portion of the left cortical kidney was immediately freeze clamped after blood collection. The cortex of the right kidney was taken for ^{31}P-NMR phospholipid determination, as will be described below.

(2) Group Mal+Gly: Gly protection against Mal-induced renal injury. The experimental protocol was identical as in Group Mal, except that rats were given 2% Gly in their drinking water starting 48 hours before first injection of Mal and continuing throughout the duration of the experiments as outlined above.

(3) Group IFO: IFO-induced renal injury. Administration of 1.6 g/m^2/day (~40 mg/kg/day) to cancer patients resulted in renal damage despite co-administation of mesna [33]. The time course of enzymuria and proteinuria achieved a peak between the third and sixth day of IFO treatment [32, 33]. Therefore, in the current study rats were given IFO (Mead Johnson, prepared as 25 mg/ml Stock solution) via i.p. injection, 50 mg/kg daily for five days, to induce nephrotoxicity. Individual rats were kept in metabolic cages with free access to Purina chow and drinking water. Urine was collected daily. On the fifth day (~120 hr) rats were anesthesized, blood samples were taken, and the kidney perfused for morphologic study or freeze clamped (as above) for biochemical measurements. Urinary parameters as reported in the **Results** section represent a collection taken approximately 16 hours before rats were sacrificed.

(4) Group IFO+Gly: Effect of Gly on IFO-induced renal injury. The experimental protocol was identical to the IFO group, except that the drinking water was supplemented with 2% Gly starting 48 hours prior to the first daily injection of IFO, as described above.

(5) Group CON: Control group. A separate group of rats was kept in metabolic cages with and without 2% Gly in their drinking water. The animals were injected with corresponding volumes of 0.9% NaCl (0.5 ml) daily for two days, or five days as a control for Mal or IFO induced nephrotoxicity, respectively. Urine samples were collected for approximately 16 hours before the rats were sacrificed. Blood samples and handling of the kidney were done as outlined above.

Analytical methods

Plasma and urine measurements. Plasma and urine content of HCO_3, Na^+, K^+, Cl^-, PO_4^{3-}, glucose, urea and creatinine were determined in the clincal chemistry laboratory of Children's Hosptial of Philadelphia, according to the protocols and methodology for the Ektachem Clinical Chemistry Slides. Amino acids were measured in deproteinized plasma using a Varian 5060 system and precolumn derivatization with O-phthalaldehyde and fluorescent detection as previously described [47]. In many cases, comparison analyses were performed in the clinical laboratory using a Beckman Amino Acid Analyzer.

Urinary metabolic profile. Urinary organic acids, including lactate, pyruvate, citrate, α-keto-glutarate, succinate and acetate were determined either with ^{1}H-NMR or GC analysis as follows:

Proton NMR spectroscopy was performed at 400MHz on the Bruker AM 400 wide-bore spectrometer. Urine samples (400 μl) were analyzed in a 5 mm tube under standard quantitative conditions (45° pulse, 8 s repetition time and water saturation during the relaxation delay, 0.256 Hx/PT digital resolution). An external standard consisting of a capillary tube containing a solution of trimethylsilylpropionic acid (TSP) in D_2O was introduced in the NMR tube, and had a triple function: the deuterium resonance was used for the lock, and the TSP resonance served as a chemical shift reference as well as for quantitation of metabolites.

In many cases, the levels of organic acids determined by ^{1}H-NMR were verified using conventional GC analysis of trimethylsilyl (TMS) derivatives as follows: A volume of urine equivalent to 200 μg creatinine was treated with methoxyamine to stabilize the oxo acids. Subsequently, the pH was adjusted to 1 to 2 and the urine extracted with 3 × 2.5 ml of ethylacetate. The ethylacetate was evaporated under a stream of nitrogen and the samples were derivatized in 150 μl of ethylacetate and 150 μl of BSTFA/TMCS (99:1; Pierce Chemical Co., Rockford, IL, USA). Analysis of the organic acids was carried out on a Hewlett-Packard 5890 gas chromatograph equipped with a split injection system (50:1), a flame ionization detector and a 7673 Autosampler. Separation was performed on a 5% phenylmethylsilicone capillary column and identification was achieved by comparison of the retention times to those of standard compounds. Chromatographic data were plotted and integrated using a Hewlett-Packard ChemStation. Calculation of the relative quantity of each compound was accomplished by the internal standard method using

undecanedioic acid. In addition, urinary amino acids were determined using Beckman Amino Acid Analyzer or HPLC and O-phthalaldehyde derivatives as indicated above.

Measurements on freeze clamped renal cortex. The frozen tissues were pulverized and extracted with 10% (wt/vol) perchloric acid (PCA). The concentration of amino acids, GSH, GSSG, and ATP as well as the inorganic and organic phophate were measured on a neutralized extract.

Amino acid levels were determined using HPLC and O-phthalaldehyde as described above. Glutathione (GSH) concentration was determined with reverse phase liquid chromatography of the monobromobimane (Thiolyte) derivative as previously described [48]. For determination of oxidized glutathione (GSSG), 100 μl of the cortical PCA extract was mixed immediately after neutralization with 900 μl of 10 mM N-ethylmaleimide (NEM) in 100 mM potassium phosphate buffer (pH 6.5). GSSG was separated from NEM and NEM-GSH adducts by passing the 1 ml mixture of NEM-tissue extract through a C_{18} cartridge, washed with 1 ml of potassium-buffer. GSSG was determined as GSH in the combined eluates following addition of DTT and the Thiolyte derivative [48]. A standard curve with a known concentration of GSSG was prepared simultaneously.

ATP was determined either fluorometerically using the method of Lamprecht and Transchold [49] or using HPLC analysis with a Beckman Model 126 system equipped with a Beckman UV detector set at 254 nm. System control and data acquisition were performed using a Beckman-Gold System version 8.10 [50]. The ATP level was also obtained from ^{31}P-NMR analysis of the cortical PCA-extract (as indicated below). In most cases good agreement was observed between the three independent methods of ATP determination and only the levels obtained by HPLC analysis are reported in the current study.

The inorganic and organic-phosphate compounds of freeze clamped renal cortex were determined using ^{31}P-NMR analysis obtained at 161.98MHz using a Bruker AM400 wide bore spectrometer equipped with an Aspect 3000 data system and a 10 mm BB probe. The lyophilized PCA extracts of renal cortex were dissolved in 1 ml of a 50 mM solution of EDTA in H_2O, pH 8.0. The solutions were then transferred to a 1 ml microcell inserted in a 10 mm NMR tube containing 3 ml of D_2O for the field/frequency lock. The vertical position of the microcell is carefully adjusted in the NMR tube so as to be in the center of the RF observe coil to maximize field homogeneity and sensitivity. Shimming is accomplished by observing the proton free induction decay (FID) signal of the sample (essentially H_2O in the microcell). A long capillary containing a solution of methylene diphosphonate (MDP) in EDTA is introduced into the microcell for the purpose of chemical shift reference and quantitation of the metabolites. The capillary was regularly calibrated against solutions of known concentration of phosphorylcholine (PC), and phosphorylethanolamine (PE).

Quantitative ^{31}P spectra were obtained nonspinning at 4°C using the "inverse gate" pulse sequence with M-LEV17, CPD turned on during acquisition only. Other acquisition parameters were as follows: sweep width 13000 Hz, 32 k data points, 45° pulse flip angle and 4 second repetition rate. Chemical shifts were referenced to 85% H_3PO_4 using the chemical shift of external MDP at 16.7 ppm as secondary reference. Before Fourier transformation, the FID was zero filled to 64 k and a Lorentz-to-Gauss lineshape transformation was applied to provide resolution enhancement. This filter narrowed the lineshape at the base of the peak allowing a better definition of the integral limits without altering the area under the resonance line.

Measurement of cortical phospholipid composition. The cortical tissue was removed as described above and lipid was extracted with 6.6 ml of methanol:chloroform:water (4:2:0.6; vol:vol:vol). After vortexing and 15 minutes on ice, an additional 2 ml of chloroform were added. The sample was vortexed and then 2 ml of 2 M KCl were added. After vortexing, the chloroform layer was separated by centrifugation, dried under N_2 gas and kept for ^{31}P-NMR analysis.

^{31}P-NMR analyses were performed as previously described [51, 52]. Briefly, the dried tissue extract was redissolved in the NMR solvent mixture consisting of 1 part of $CdCl_3$, 1 part of $CHCl_3$ and 1 part of MeOH/EDTA. The mixture of MeOH/EDTA contains MeOH and a 0.2 M solution of K-EDTA in H_2O (4:1). K-EDTA is prepared from the free acid by neutralization to pH 6.0 with high purity KOH. A thin sealed capillary tube containing a diluted solution of H_3PO_4 is then inserted to allow the quantitation of the different phospholipids. The capillary was calibrated before and after each series of experiments using a solution of known concentration of L-phosphatidylcholine dimyristoyl. Depending on the amount of tissue available, the volume of solvent for the NMR sample was either 0.6 ml for 5 mm tubes or 3 ml for 10 mm tubes.

The "inverse-gate" pulse sequence was used to obtain quantitative spectra. The composite pulse decoupling (CPD) was turned on during the acquisition time only. The acquisition parameters were as follows: sweep width 1500 Hz, 8 k data points, 45° pulse flip angle, 4 second repetition rate. Chemical shifts were referenced using the resonance of phosphatidylcholine.

Calculation and statistical analysis

The rate of excretion of any given metabolite or electrolyte, U is the product of urinary excretion rate (vol, ml/hr), times concentration of the metaboltie (μmol/ml) in the same urine sample. The fractional excretion (FE) of the indicated metabolites (Table 1) was calculated by the standard formula [53]. Creatinine clearance (C_{Cr}) rate (ml/hr) was calculated as: (urine C_{Cr} × urine volume)/plasma C_{Cr} [53].

A multiple comparison was made between experimental groups using one way analysis of variance followed by the Bonferroni test to detect differences between groups. Comparison between two groups was done by the nonparametric (Mann-Whitney) test for unpaired data using Instat software package for Macintosh.

Results

Abnormalities of clearance and tubular function induced by Mal or IFO

Both Mal and IFO induced significant rises of plasma BUN and C_{Cr} (Fig. 1). BUN and C_{Cr} increased from 18.1 ± 1.2 and 0.39 ± 0.03 mg/dl, respectively, in the controls to 65.3 ± 12.1 ($N = 7, P = 0.0006$) and 0.94 ± 0.15 mg/dl ($P = 0.0006$), respectively, after Mal and 28.3 ± 1.8 ($N = 9$, $P = 0.0002$) and 0.56 ± 0.03 mg/dl ($P = 0.0006$), respectively, after IFO (Fig. 1). C_{Cr} excretion rates in the IFO-treated rats was markedly reduced to less than 60% of that in control and Mal-treated rats (Table 1), so that C_{Cr}

Table 1. Parameters of urinary metabolite excretion

Parameters	Experimental Group				
	Control	Maleate	Maleate + Gly	Ifosfamide	Ifosfamide + Gly
Urine excretion *ml/hr*	0.77 ± 0.04	1.37 ± 0.12 (P = 0.0001)	1.33 ± 0.13 (P = 0.004)	0.84 ± 0.16	0.89 ± 0.11
U_{Cr} *μmol/hr*	5.70 ± 0.29	6.14 ± 1.20	7.54 ± 1.32	3.82 ± 0.87 (P = 0.002)	5.32 ± 0.84
$U_{creatine}$ *μmol/hr*	-0-	1.25 ± 0.21	0.11 ± 0.10[a]	6.03 ± 0.58	5.09 ± 0.41
FE_{Na} %	0.02 ± 0.01	3.97 ± 1.97 (P = 0.002)	0.46 ± 0.35[a]	0.82 ± 0.44 (P = 0.039)	0.35 ± 0.19 (P = 0.122)
U_{Na} *μmol/hr*	38.5 ± 8.9	1040 ± 287 (P = 0.009)	65.6 ± 13.3[a]	103.1 ± 45	105 ± 58
FE_K %	11.1 ± 3.5	64.6 ± 14.8 (P = 0.023)	28.4 ± 6.0[a] (P = 0.09)	28.5 ± 6.6 (P = 0.016)	25.9 ± 3.8 (P = 0.033)
U_K *μmol/hr*	63.5 ± 21.7	2183 ± 553 (P = 0.03)	99.3 ± 24.4[a]	76.3 ± 17.5	75.5 ± 7.3
FE_{PO_4} %	2.3 ± 0.6	53.2 ± 10.5 (P = 0.002)	21.5 ± 5.5[a] (P = 0.002)	36.5 ± 6.1 (P = 0.001)	35.5 ± 5.1 (P = 0.002)
U_{PO_4} *μmol/hr*	2.2 ± 0.8	24.7 ± 9.8 (P = 0.002)	3.96 ± 1.1[a]	17.6 ± 2.9 (P = 0.004)	20.7 ± 2.5 (P = 0.007)
$FE_{glucose}$ %	0.12 ± 0.04	16.1 ± 5.9 (P = 0.004)	4.1 ± 1.8[a] (P = 0.01)	0.59 ± 0.16 (P = 0.03)	0.09 ± 0.01[a]
$U_{glucose}$ *μmol/hr*	1.5 ± 0.6	110.2 ± 36.7 (P = 0.002)	65.8 ± 46.3 (P = 0.005)	2.6 ± 0.9	1.6 ± 0.6
$U_{amino\ acid}$[b] *μmol/hr*	27.2 ± 8.2	333.4 ± 135.9 (P = 0.02)	165.4 ± 63.5 (P = 0.01)	234.6 ± 103.7 (P = 0.01)	61.8 ± 11.6[a] (P = 0.05)
$U_{organic\ acid}$[c] *μmol/hr*	14.9 ± 2.0	129.3 ± 19.4 (P = 0.001)	47.3 ± 10.6[a] (P = 0.006)	31.5 ± 8.6 (P = 0.02)	20.8 ± 3.2

U, Urinary excretion rate; FE, fractional excretion rate calculated as indicated under **Methods.** Values are means ± SE (N = 6 to 9). P values are compared with control using unpaired nonparameteric test. When P value is not indicated, the difference is statistically insignificant.

[a] P values are smaller than 0.05 when compared with the same experimental group without glycine

[b] Amino acids are the sum of amino-N except glycine

[c] Organic acids are the sum of lactate, pyruvate, fumarate, citrate, α-ketoglutarate, acetate and hippurate.

clearance was decreased to the same extent in both the Mal and IFO groups (Fig. 1).

Neither Mal nor IFO affected plasma Na^+, K^+, Cl^-, glucose or Ca^{2+} (Table 2). However, both HCO_3^- and PO_4^{3-} were substantially decreased following administration of Mal, which is in agreement with previous studies in rats [42–45] and dogs [37–39]. More modest decreases in both parameters of approximately 15% ($P > 0.05$) were seen after IFO (Table 2). Urine volumes were doubled in the Mal-treated animals as compared to controls, but were not significantly increased after IFO (Table 1). Mal-treated rats showed extensive urinary losses of Na^+, K^+, PO_4^{3-}, glucose, amino acids and organic acids (Table 1). Qualitatively similar, but less severe losses were seen after IFO. Creatine, which was absent in the urine of controls, was excreted by both the Mal and IFO-treated rats, but, unlike the other urinary parameters, was approximately fourfold higher after IFO, 6.03 ± 0.58 μmol/hr, than after Mal, 1.25 ± 0.21 μmol/hr (Table 1).

Effects of Gly on functional abnormalities induced by Mal and IFO

Although the plasma creatinine values of rats given 2% Gly in their drinking water beginning two days prior to Mal or IFO treatment were significantly increased as compared to controls (Fig. 1), the changes of BUN and of C_{Cr} clearance in both Mal+Gly and IFO+Gly rats were substantially and significantly less than those in the animals not given Gly (Fig. 1). Gly treatment did not affect the increases of urine volume seen after Mal, however fractional and total excretions of Na^+, K^+, PO_4^{3-} and glucose and total organic acids excretion were all significantly improved by Gly (Table 1). Aminoaciduria was also decreased, but the difference between the Mal and Mal+Gly groups did not reach significance (Table 1). Gly did not affect the decreases of creatinine or increases of creatine excretion in the IFO-treated rats, but significantly improved the Na wasting and aminoaciduria (Table 1). Gly prevented the decreases of plasma HCO^-_3 and PO_4^{3-} seen after both Mal and IFO (Table 2).

Figure 2 provides a composite analysis of urinary metabolite excretion obtained with ^{1}H-NMR. High resolution ^{1}H-NMR has been previously used for rapid multicomponent analysis in approximately 0.5 ml of urine [54, 55]. This methodology can provide early detection of biochemical changes associated with renal dysfunction [55]. In the current study, urinary ^{1}H-NMR analyses demonstrated the aminoaciduria, organicaciduria and glucosuria, main characteristics of Fanconi syndrome [25–28], and the marked attenuation produced by Gly supplementation in the pathologic urinary excretion of these metabolites (Fig. 2). The ^{1}H-NMR spectra of Figure 2 also demonstrate that rats given Gly in drinking water excreted substantially higher amounts of urea compared with rats without Gly as well as the changes of Crn and creatine excretion that were detected chemically (Table 1). Furthermore, the patterns of excretion of individual organic acids show that Mal treatment was accompanied by decreased citrate excretion associated with elevation of α-keto-glutarate, lactate, succinate, acetate and pyruvate. Gly reversed these changes toward the urinary profile of control rats (Fig. 2). Similar changes

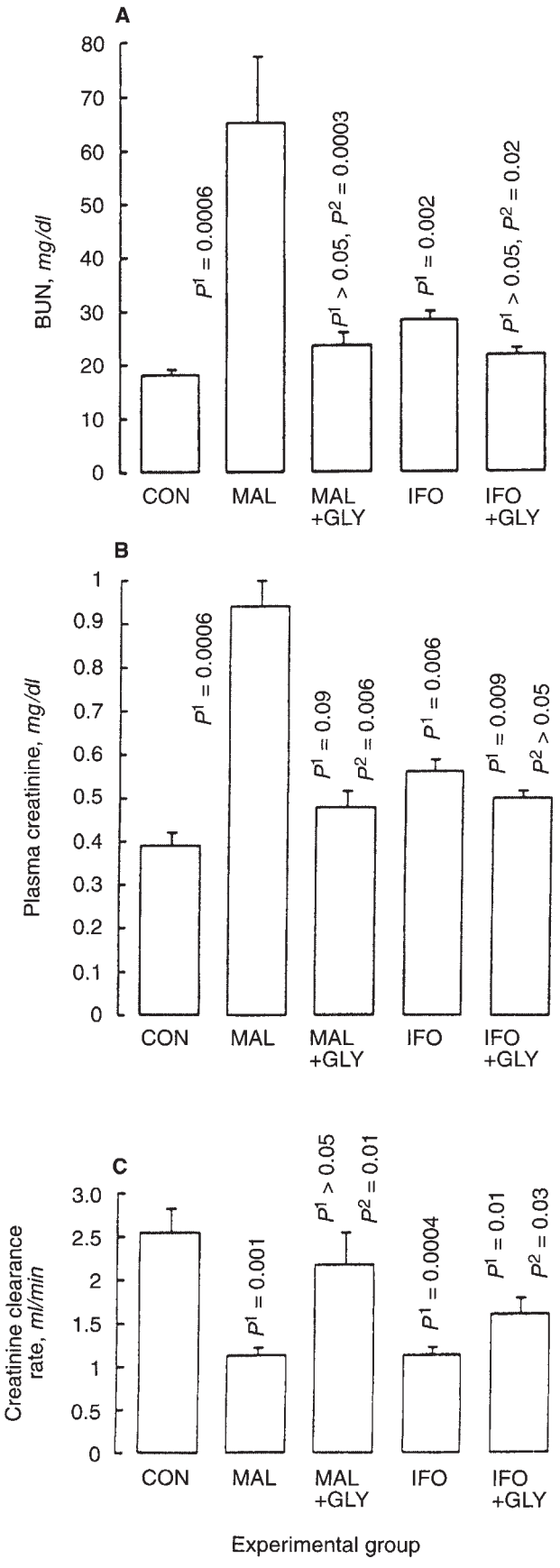

Fig. 1. *Concentration of urea-N in the blood (A); plasma creatinine (B) and creatinine clearance rate (C) obtained from: (a) control group (CON, N = 7); (b) rats treat with a single i.p. injection of 2 mmol/kg maleate, daily for two days (MAL, N = 7); (c) Rats treated with maleate as indicated above with 2% (wt/vol) of glycine in their drinking water starting, 48 hours prior to first Maleate injection (MAL+GLY, N = 7); (d) rats treated daily for five days with a single i.p. injection of 50 mg/kg IFO (IFO, N = 9); and (e) same as IFO group with supplementation of 2% glycine in their drinking water, starting 48 hours prior to first injection of IFO (IFO+Gly, N = 8).* In Maleate treated rats BUN and plasma C_{Cr} were measured at 24 hours after the second injection of Mal. In IFO and IFO+GLY, BUN and C_{Cr} were measured on the fifth day, at approximately 120 hours after the first injection of IFO. Urinary measurements of creatinine for calculation of clearance rates were performed on urine collected for approximately 16 hours before operation of the animal as indicated in the **Methods** section. Values are means ± SEM; p^1 is compared with control and p^2 compared with the same experimental group without glycine, using unpaired non-parametric test.

were observed in most urinary organic acids following IFO or IFO+Gly treatment (Fig. 2).

Structural effects of Mal and IFO and their modification by Gly

The rats treated with Mal developed proximal tubule cell necrosis that was confined to the cortex and was most prominent in the subcapsular areas (Fig. 3 b, e). The Mal + Gly group was remarkably protected against this necrotic damage, which was entirely absent in some animals and confined to occasional tubule profiles in the others (Fig. 3 c, f). IFO-treated rats did not develop necrotic tubular damage; brush border abnormalities after IFO were seen with equal frequency irrespective of the presence of Gly (not shown).

Plasma and cortical levels of Gly – Relationship to tubular protection

Table 3 shows insignificant differences in plasma Gly between control, Mal or IFO groups without Gly supplementation. However, consumption of 2% Gly in the drinking water elevated plasma Gly by approximately threefold, reaching 0.76, 0.81 and 0.55 mmol/liter in control+Gly, Mal+Gly and IFO+Gly groups, respectively (Table 3). There was little change in the levels of other plasma amino acids (data not shown).

In the freeze clamped renal cortex, both Gly and serine levels were increased significantly in the control group supplemented with 2% Gly in drinking water compared to control without Gly (Table 3). In Mal and IFO treated rats without supplementation of Gly, the tissue levels of Gly, serine and alanine were depleted significantly compared with untreated rats. However, following Gly supplementation in the drinking water the tissue levels of Gly, alanine and serine were increased significantly compared with Mal and IFO treated rats without Gly (Table 3). In the Mal+Gly group, the tissue Gly level was 5.7 ± 0.8; Ala, 0.75 ± 0.1 and Ser 0.74 ± 0.06 μmol/g wet wt. In IFO+Gly treated group these values were 3.9 ± 0.7; 0.89 ± 0.2 and 0.71 ± 0.1 μmol/g wet wt for Gly, alanine and serine, respectively. Although the Gly level in the IFO+Gly group was similar to the control group without supplementation of Gly, this level was significantly lower than that of the Con+Gly group (Table 3).

Table 2. Biochemical parameters in arterial plasma

Experimental group	Na^+	K^+	Cl^-	HCO_3	Glucose	Ca^{2+}	PO_4
	mmol/liter					*mg/dl*	
Control	147.3 ± 2.6	5.2 ± 0.2	100 ± 1.5	22.8 ± 0.5	211 ± 9.7	10.5 ± 0.4	7.1 ± 0.7
Maleate	156.8 ± 3.7	5.0 ± 0.4	106 ± 4.5	16.2 ± 1.6 $P = 0.004$	202 ± 11.7	9.7 ± 0.8	6.1 ± 1.0 $P = 0.007$
Maleate & Gly	152.8 ± 2.7	4.9 ± 0.3	103 ± 2.5	19.1 ± 1.2	219 ± 11.6	9.6 ± 0.5	6.9 ± 0.3
Ifosfamide	152.0 ± 2.2	4.8 ± 0.2	102 ± 1.6	21.1 ± 1.1	206 ± 4.4	9.6 ± 0.2	6.4 ± 0.2
Ifosfamide & Gly	146.5 ± 1.5	4.6 ± 0.1	102 ± 1.2	21.0 ± 1.0	206 ± 10.1	10.2 ± 0.2	7.3 ± 0.2

Values are means (N = 6 to 9) ± SE and P values are compared with control group using unpaired nonparametric test. When P value is not indicated, the difference is not statistically significant.

Induction of renal cortical injury and prevention by Gly – Relationship to energetic state and antioxidant capacity

Cellular energetic state and oxidant stress as reflected by cellular ATP and GSH/GSSG levels are major metabolic disturbances that occur during Mal induced cellular injury [28, 39, 56–60]. To explore the relationship between Mal or IFO induced tubular injury and the renal cortical energetic state, glutathione metabolism, and Gly cytoprotection, we measured cellular Pi, ATP, GSH and GSSG in the freeze clamped cortical tissue of all experimental groups. Table 4 demonstrates that, following Mal or IFO administration, tissue ATP and GSH levels decreased by approximately 50%. Supplementation with Gly completely reversed the decrease in ATP level of Mal treated rats, but did not affect the change in IFO treated rats. Table 4 also shows that the cellular level of inorganic phosphate (P_i) dropped by approximately 30% and 20% in Mal and IFO treated rats, respectively compared with control. This decrement in P_i was reversed in Mal+Gly but not in IFO+Gly groups.

Gly did not significantly affect the depletion of tissue GSH in either the Mal or IFO treated groups although there was an upward trend in the IFO+Gly group so that it no longer differed from either IFO alone or the control. The levels of oxidized glutathione, that is, GSSG, were approximately fourfold and sixfold higher in Mal and Mal+Gly, compared with control groups and were 2-fold and 14-fold greater than controls in the the IFO and IFO+Gly groups, respectively. The ratios of GSSG/GSH were 0.006, 0.07 and 0.13 in control, Mal and Mal+Gly groups, respectively. In rats treated with IFO, the GSSG/GSH ratio was 0.04 and 0.27 in IFO and IFO+Gly groups, respectively (Table 4).

Induction of renal tubular injury and protection by Gly – Relationship to cortical phospholipid composition and degradation

Table 5 summarizes the ^{31}P-NMR analysis of the chloroform-methanol extracts of renal cortex and Figure 4 provides a representative ^{31}P-NMR spectra. In Mal-treated rats, there were significant decreases of approximately 30% of phosphatidyl-ethanolamine (P_tE) and phosphatidyl-serine (P_tS), while decreases of about 20% in phosphatidyl-choline (P_tCh), sphingomyelin (SP) and cardiolipin (Cl) relative to the control group were not statistically significant. In contrast, although the values tended to be lower than those of controls, the Mal+Gly-treated rats did not show significant decreases in any phospholipid. In CON+Gly, there was no difference in the tissue levels of PtE and/or P-serine compared with control group without Gly (data not shown). In IFO treated rats, the 24% decrease of P_tE compared with control was significant ($P = 0.02$) and this effect was completely prevented by Gly. Changes in other phospholipids in the IFO-treated rats were in the range of 10% to 15% and, although these were also completely prevented by Gly, none of the differences reached statistical significance.

Discussion

In the present studies, we have examined a classical experimental form of Fanconi syndrome, Mal nephrotoxicity [37–45], and a less well characterized, but clinically relevant lesion induced by IFO [29–34]. In both cases, oral supplementation of Gly in a fashion that moderately increased plasma concentrations was highly beneficial with respect to both glomerular function as well as the tubular reabsorptive deficits.

The maleic acid model of FS was the first to be recognized and the most extensively used to elucidate the physiological, morphological and biochemical correlates [37–45]. The use of ifosfamide to induce FS in rats has not previously been reported. Although, the initial reports of ifosfamide induced-nephrotoxicity are from 1970's [reviewed in 30], there is no informations regarding the biochemical perturbation as well as the mechanism(s) of kidney injury following ifosfamide treatment. The current study demonstrates that ifosfamide induced renal dysfunction in rats. This disorder resembles maleate-induced Fanconi syndrome in rats including, a remarkable urinary excretion of amino and organic acids, glucose and phosphate as well as elevation of plasma C_{Cr} and BUN and decreased creatinine clearance rate (Figs. 1, 2 and Tables 1, 2).The current observationts suggest that rats can be used as model to elucidate the mechanism(s) responsible for renal dysfunction that occurs secondary to ifosfamide treatment. Furthermore, the current demonstration that oral supplementation of glycine has minimized renal impairment associated with IFO treatment, may have a clinical implication for the prevention of nephrotoxicity in cancer patients.

Prior *in vivo* studies of glycine cytoprotective action in rats in which serum levels were reported [16, 17] employed infusions of Gly as high as 75 mg/100 g body wt per hour that increased plasma levels 20- to 40-fold to 3 to 4 mM, that is, concentrations that are fully cytoprotective *in vitro*. During ischemia-reflow and contrast-induced lesions, Gly administration in this fashion served to aggravate rather than suppress tubule cell damage, possibly as a result of increased workload and decreased tissue oxygenation [16]. The present studies used 2% Gly in drinking water starting 48 hours prior to Mal or IFO treatment. On the average, daily intake of water by the rats was approximately 30 ml, so that they consumed approximately 25 mg Gly/hour or 10 mg/hr/100 g body wt (the average body wt was 250 g). This yielded moderate, that is, two- to threefold, but, sustained and apparently highly effective increments in plasma Gly. Dose dependence studies of Gly

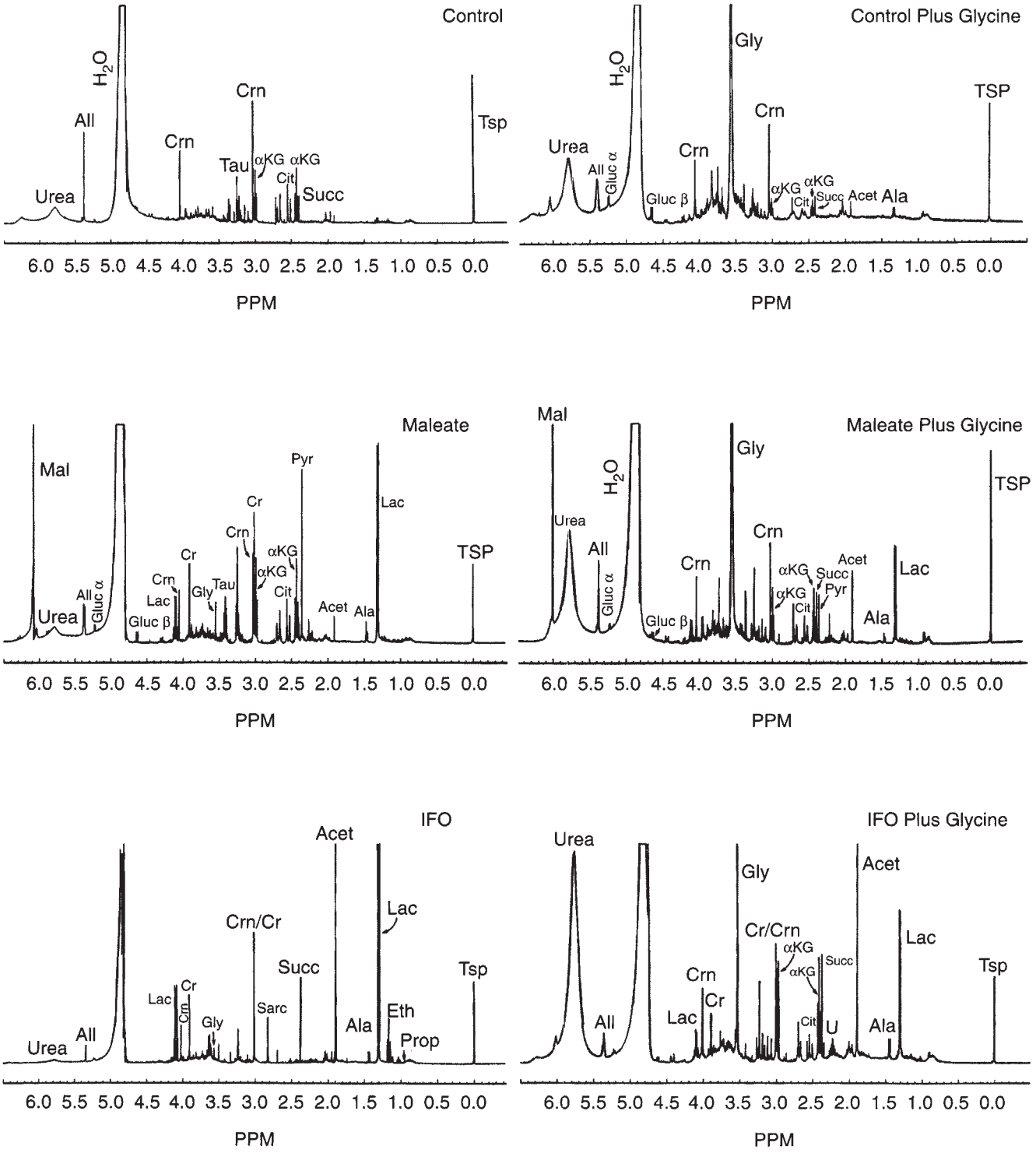

Fig. 2. *^{1}H-NMR spectra obtained from urine collected for 16 hours before sacrifice of the animal as indicated in the* ***Methods*** *section.* Control, intact rats; control plus glycine, intact rats with glycine in drinking water; Maleate, rats treated with Mal without glycine or with glycine; IFO, treated rats without glycine or with glycine. Experimental details per each study groups are as indicated in the legend of Figure 1. Acquisition parameters for ^{1}H-NMR spectra are as described in the Methods section. Abbreviations are: TSP, trimethylsilyl proponic acid serves as a reference for chemical shifts and as standard for quantitation, its concentration being 1 mM in the sample; Eth, ethanol; Lac, lactate; Ala, alanine; Acet, acetate; Pyr, pyruvate; Succ, succinate; α-KG, alpha-ketoglutarate; Cit, citrate; Sarc, sarcosine; Crn, creatinine; Cr, creatine; Tau, taurine; Gly, glycine; Gluc α, glucose α anomeric proton; Gluc β, glucose β anomeric proton; All, allantoin; Mal, maleate.

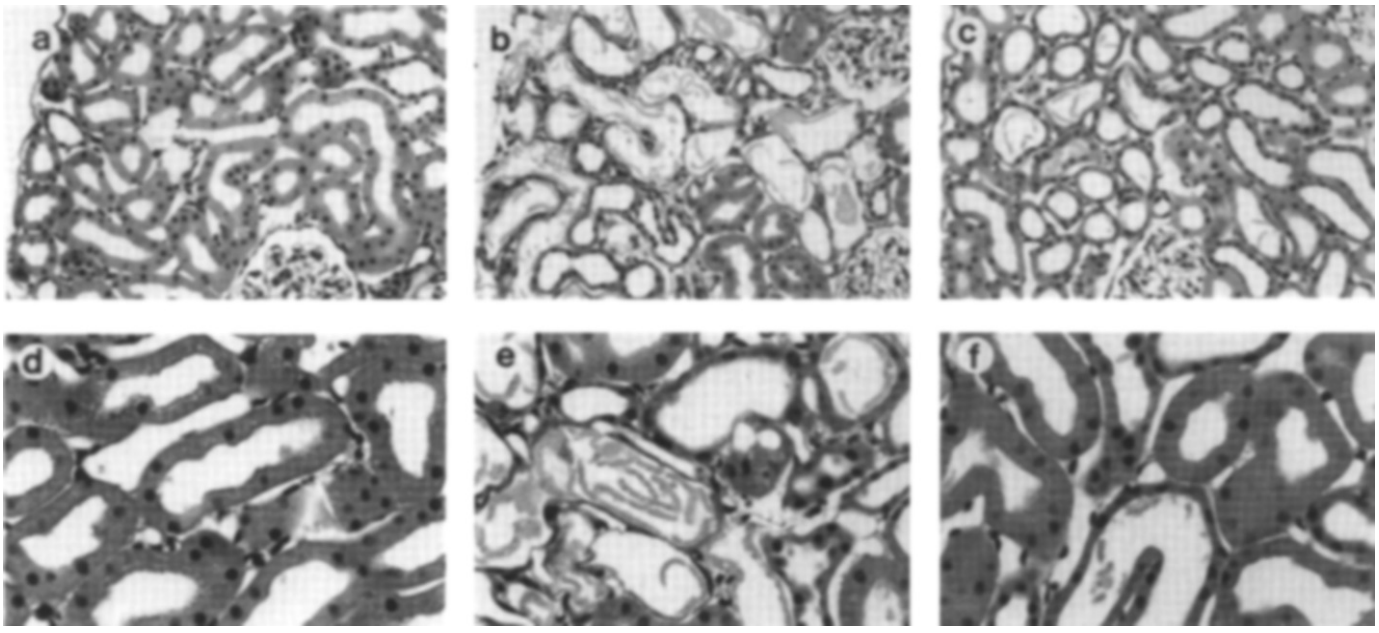

Fig. 3. *Light micrographs of hematoxylin and eosin-stained sections of perfusion-fixed kidneys from control (***a, d***), Mal (***b, e***), and Mal+GLY (***c, f***) groups showing the subcapsular areas where Mal-induced necrotic damage was most prominent.* In each panel, the renal capsule is on the left. It can be seen in the upper left corner of each of the lower power pictures (a-c). Panels a-c are at ×130; d-f are the corresponding conditions at ×260.

Table 3. Amino acid levels in plasma and kidney cortex

Experimental group	Plasma glycine *nmol/ml*	Kidney tissue amino acid *μmol/g wet wt*		
		Glycine	Alanine	Serine
Control	298.1 ± 12.9 $N = 8$	3.1 ± 0.2 $N = 12$	0.86 ± 0.08 $N = 12$	0.78 ± 0.07 $N = 12$
Control & Gly	764.0 ± 54.6 $N = 4$; $P = 0.004$	6.1 ± 1.1 $N = 3$; $P = 0.03$	0.8 ± 0.1 $N = 3$; $P > 0.05$	1.2 ± 0.2 $N = 3$; $P = 0.04$
Maleate	283.3 ± 37 $N = 7$; $P > 0.05$	2.2 ± 0.3 $N = 7$; $P = 0.05$	0.46 ± 0.06 $N = 5$; $P = 0.005$	0.53 ± 0.06 $N = 7$; $P = 0.02$
Maleate & Gly	815.1 ± 45.0 $N = 9$; $P = 0.006$	5.7 ± 0.8 $N = 7$; $P = 0.02$ $P^2 = 0.001$	0.75 ± 0.07 $N = 8$; $P > 0.05$ $P^2 = 0.01$	0.74 ± 0.06 $N = 5$; $P = 0.03$ $P^2 = 0.05$
Ifosfamide	269.1 ± 19.8 $N = 10$; $P > 0.05$	2.1 ± 0.3 $N = 9$; $P = 0.01$	0.77 ± 0.08 $N = 8$; $P > 0.05$	0.58 ± 0.04 $N = 8$; $P = 0.02$
Ifosfamide & Gly	551.7 ± 23.6 $N = 10$; $P = 0.0005$, $P^2 = 0.008$	3.9 ± 0.7 $N = 5$; $P > 0.05$ $P^2 = 0.04$	0.89 ± 0.17 $N = 5$; $P > 0.05$	0.71 ± 0.09 $N = 5$; $P > 0.05$, $P^2 = 0.06$

Values are means ± SE. P values are compared with control and P^2, compared with the same experimental study without glycine using unpaired nonparametric test.

cytoprotection in isolated cell systems also show that substantial, albeit incomplete, benefit can be derived from similar increments of medium Gly concentrations, although energy-dependent cellular uptake and concentrative mechanisms were highly suppressed in *in vitro* systems studied to date [1–3]. This differs from the situation during *in vivo* studies such as the present one, where continued delivery of Gly and operation of concentrative mechanisms allowed full restoration of decreased tissue Gly in the Mal and IFO-treated rats to supranormal concentrations approaching those in control animals supplemented with Gly, despite the moderate increments in plasma levels (Table 3). It is also relevant in this regard that the tissue levels of Gly measured in the unprotected animals likely underestimate the degree of Gly depletion in the most severely affected cells because of the heterogeneity of the insult. The observed urinary excretion of excess urea in the Gly-treated animals in the present study (Fig. 2) likely reflects metabolism of much of the supplemented Gly to urea-N by the liver.

Mal, as used in the present studies, produced the most severe tubule structural abnormalities and functional changes, both of which are consistent with prior reports [25, 43, 60, 61]. The dramatic improvement induced by Gly in all of the functional parameters almost certainly reflects the nearly complete structural preservation. The findings with IFO are more complex in that proximal tubule necrosis did not develop and tubular function abnormalities tended to be less severe, yet Gly still improved several of them to a significant degree. Information possibly related to this latter observation has been reported by Baines, Shaikh and Ho [62] in the isolated perfused kidney, where Gly not only protected against the hypoxic, necrotic damage to the

Table 4. Renal cortical Pi, ATP and glutathione

Experimental group	Pi[a]	ATP	GSH[b]	GSSG
		nmol/g wet wt		
Control	11.1 ± 1.2	1553 ± 240	1375 ± 226	9.1 ± 4.6
Maleate	7.9 ± 1.2	567 ± 145	575 ± 86	37.5 ± 3.6
P	0.08	0.01	0.002	0.03
Maleate+Gly	11.9 ± 2.9	1584 ± 232	521 ± 85	68.4 ± 18.5
P	> 0.05	> 0.05	0.009	0.03
Ifosfamide	9.1 ± 1.2	821 ± 139	452 ± 148	17.6 ± 9.5
P	0.15	0.01	0.008	> 0.05
Ifosfamide+Gly	8.5 ± 1.2	865 ± 162	557 ± 145	148.3 ± 67
P	0.09	0.09	0.02	0.01

Values are means (N = 4 to 6), ± SE. P values are compared with control using unpaired nonparametric test.

[a] Pi was determined using ^{31}P-NMR analysis of renal cortical PCA extract and expressed as μmol/g wet weight

[b] Reduced glutathione, that is, total glutathione minus oxidized glutathione (GSSG)

medullary thick ascending limb characteristic of that system, but also improved functional parameters of the proximal tubules, where structural damage is not typically seen [62].

The metabolic measurements suggest that Gly may have been acting via its membrane cytoprotective effect seen during a variety of insults *in vitro* [1–14] as well as to directly counteract cellular effects of the toxins, and, possibly, to promote repair. Mal, acrolein (see below) or their CoA complexes (maleyl-CoA) can bind to SH groups of glutathione and other cellular peptides, including membrane proteins [58, 59, 63]. Since GSH and the glutathione redox cycle are a main defense mechanism against free radical-induced cellular injury [64], the latter process may further complicate the lesions and information that one such effect, lipid peroxidation, contributes to Mal nephrotoxicity has been reported [58, 59]. The glutathione measurements in Table 4 provide evidence for an action distal to this primary toxic effect of the agents, since Gly did not consistently ameliorate the decreases of GSH and increases of GSSG seen during both types of injury. The membrane cytoprotective effect of Gly seen *in vitro* is not mediated by preservation of GSH [1, 4, 66] or antioxidant effects [10].

Maintenance of cellular ATP, irrespective of the mechanism, appears to play a critical role in the severity of the insult produced by Mal [42, 65]. Exogenous ATP, which can substantially increase tissue levels [68, 69], has been reported to ameliorate the lesion [65]. Protection by Gly during the Mal model in the current studies was accompanied by complete preservation of normal ATP levels. The membrane cytoprotective effects of Gly are not mediated by preservation of ATP [3, 9, 10], although higher levels of ATP during Gly cytoprotection can occur as a consequence of general maintenance of cellular integrity during proximal tubule insults that are not produced by primary, maximal suppression of mitochondrial function [66, 67]. That ATP was so completely preserved by Gly treatement during Mal-induced injury despite the well documented effects of Mal to inhibit TCA cytcle function [40–43] suggests an effect of Gly to directly mitigate the mitochondrial toxicity of Mal. This conclusion is supported by the lower levels of α-ketoglutarate, acetate, pyruvate, and lactate excretion in the Mal+Gly treated rats since these metabolites would be expected to accumulate as a result of Mal-induced CoA depletion [42]. Inhibition of the TCA cycle by Mal is considered to be related to formation of maleyl-CoA and depletion of intramitochondrial free coenzyme A [42], which is required in the oxidation of α-ketoglutarate to succinyl CoA [41, 42]. The mechanism of IFO-induced renal impairment is obscure, but it has been suggested that nephrotoxicity is secondary to acrolein and/or chloroacetaldehyde, metabolites of IFO [30, 35]. Acrolein, an alkylating agent like Mal can also bind to CoA, forming an acrolein-CoA complex as a trapping system, thus, potentially inhibiting TCA-cycle activity. A conceivable mechanism by which Gly could directly antagonize mitochondrial toxicity would be by interacting with acrolein or Mal intracellularly to form a Schiff base or a conjugate, thereby minimizing the removal of CoA and maintaining normal activity of the TCA cycle. Whether the same occurs intracellularly and alters toxic effect of Mal or acrolein remains to be tested. The prevention of ATP depletion after Mal by Gly (Table 4) is certainly consistent with this mechanism.

Losses of intracellular Pi, which is necessary for normal ATP turnover [70, 71], have also been proposed to play a major role in development of maleate-induced nephrotoxicity [39, 44, 45]. A substantial decrease of Pi occurred after Mal in the present studies and was entirely prevented in the Gly-treated animals. The decrease of Pi after IFO was less severe and was not modified by Gly. Gly has been shown to decrease the toxicity of phosphate depletion to isolated tubules, but, in that setting does not affect the extent of either P_i or ATP depletion [5].

Decreases of tissue phospholipid content occurred after Mal and, to a lesser extent, after IFO, in the unprotected animals (Table 5). These changes were not simply due to loss of total membrane mass and resulting washout of debris from necrotic cells because they were selective for individual phospolipids and exceeded the extent of necrosis. Decreases of PtE were most prominent with both toxins. This finding is of particular interest in view of recent observations that Ca^{2+}-independent phospholipases selective for plasmalogen-containing PtE play major roles in the breakdown of proximal tubule phospholipids during severe ATP depletion states [72, 73]. Gly-treated animals were virtually completely protected from the phospholipid losses. Inhibition of phospholipid breakdown and free fatty acid accumulation are neither primary effects of Gly nor required for its membrane cytoprotective actions [3, 73, 74]. Thus, the preservation of phospholipids in the present study likely reflects in large part the maintenance of overall cell integrity and metabolic function. However, the data suggest an additional beneficial effect of Gly in that phospholipid content was also maintained in the IFO-treated rats where Gly did not improve ATP levels and necrosis did not occur. An explanation for this action of Gly may be found in its effect to increase tissue levels of serine [75], which is required for synthesis of PtE as well as of PtS [76]. Because serine is the precursor of PtE and PtS, the decreased levels of Gly and serine in Mal or IFO treated rats (Table 3), when combined with the change in PtE and PtS, suggest that Gly may furnish the serine needed in the *de novo* synthesis of phospholipids. However, further studies are required to examine this hypothesis.

It is of interest that creatine excretion was increased after both Mal and IFO, but the change was particularly marked after IFO and was accompanied by significantly decreased creatinine excretion. Gly corrected the creatine losses after Mal and normalized creatinine excretion after IFO. With Mal, the effect of Gly to decrease creatine excretion is probably a result of the protection of tubule structure and function. With IFO, the normalized

Table 5. ^{31}P-NMR analysis of renal cortical phospholipids

Experimental group	P-choline[a]	P-inositol[a]	P-ethanolamine[a]	P-serine[a]	Sphingomyelin	Cardiolipin
	μmol/g wet wt					
Control (N = 7)	11.2 ± 0.8	1.7 ± 0.1	7.1 ± 0.4	2.1 ± 0.2	4.4 ± 0.3	2.5 ± 0.1
Maleate (N = 4)	9.1 ± 1.8 (81%)	1.6 ± 0.2 (94%)	5.1 ± 0.9 (71%; P = 0.4)	1.4 ± 0.3 (66%; P = 0.07)	3.3 ± 0.6 (75%)	2.2 ± 0.9 (88%)
Maleate & Gly (N = 4)	9.3 ± 0.9 (83%)	1.6 ± 0.1 (94%)	6.2 ± 0.4 (87%)	1.9 ± 0.1 (90%)	3.9 ± 0.5 (87%)	2.1 ± 0.3 (85%)
Ifosfamide (N = 4)	9.5 ± 0.9 (85%)	1.7 ± 0.1 (100%)	5.4 ± 0.5 (76%; P = 0.02)	1.9 ± 0.3 (90%)	4.1 ± 0.6 (93%)	2.2 ± 0.1 (88%)
Ifosfamide & Gly (N = 4)	10.4 ± 1.1 (93%)	1.8 ± 0.2 (105%)	7.1 ± 0.6 (100%)	2.2 ± 0.3 (100%)	4.9 ± 0.2 (111%)	2.9 ± 0.3 (116%)

Values are mean ± SE. Numbers in parenthesis are % of control levels. P values are compared with control group using unpaired nonparametric test. When the P value is not indicated, the difference is statistically insignificant.

[a] P, *phosphatidyl*

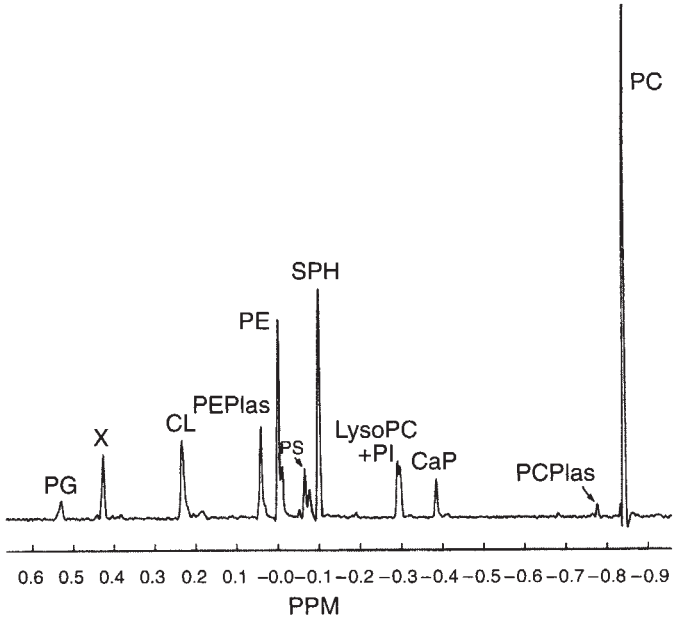

Fig. 4. *Representative ^{31}P-NMR spectra of renal cortical chloroform-ethanol extract of phospholipids.* Spectra have obtained as detailed in the **Methods** section. The quantitation of various phospholipids was based on the integral of H_3PO_4 resonance in sealed capillary (Cap). Abbreviations are: PC, phosphatidylcholine; PCPlus, PC-plasmalogen; PI, phosphatidylinositol; SPh, sphingophospholipid; PS, phosphatidylserine; PE, phosphatidylethanolamine; CL, cardiolipin; X, unknown; PG, phosphatidylglycerol.

creatinine excretion is unlikely to be solely due to improved renal function, since the lesion was subacute at that point and net excretion was probably at steady state. Instead, the increased creatinine excretion despite persisting abnormal loss of creatine suggests an additional effect of glycine to improve abnormalities of creatine metabolism in muscle or synthesis in the liver that are secondary to toxicity arising from the alkylating effects of IFO. This improvement could be due to effects of glycine on IFO-induced injury to liver and/or muscle, the main sites of creatine synthesis and metabolism [77].

In conclusion, the data demonstrate that oral supplementation with Gly is sufficient to correct Gly depletion in the kidney that develops during two toxic lesions causing the Fanconi syndrome, and markedly diminish both structural damage and functional abnormalities in both cases. This effect has characteristics suggestive of the membrane cytoprotective action of Gly that has been observed during multiple forms of injury *in vitro*, but the studies also suggest actions of Gly on the primary toxicity of the agents and on metabolic events which may be important for repair processes. It deserves further investigation both for the insight it can provide into actions of Gly *in vivo* and the possibilities of impacting on the clinically relevant IFO lesion. The effectiveness of oral glycine and of rather moderate increases in circulating glycine levels against ifosfamide toxicity shown here and prior data suggesting benefit during cisplatin treatment [15], albeit by different mechanisms, suggest that further consideration may be given to clinical application of this approach to reducing the toxic effects associated with cancer chemotherapy.

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