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ORGAN TOXICITY AND MECHANISMS

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Adenine nucleotide and calpain inhibitor I protect against atractyloside-induced toxicity in rat renal cortical slices in vitro

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Abstract Atractyloside is a compound with a documented nephrotoxicity. It induces renal tubular necrosis at high doses and apoptosis at lower doses. This study investigates the potential protective effect of some chemical agents against atractyloside-induced nephrotoxicity in vitro using the precision-cut rat renal cortical slices obtained from kidneys of Wistar rats. For co-incubation experiments, slices were incubated for 3 h at 37°C on a rocker platform with various chemical agents: ADP (5 mM), calpain inhibitor I (CPI, 1 mM), stevioside (STV, 2.5 mM) or probenecid (PRB, 2.5 mM) in the presence or absence of atractyloside (2 mM). For pre-incubation experiments, slices were incubated with the same chemical agents for 1 h before exposure to atractyloside. The nephrotoxic effects of atractyloside (2 mM) alone were manifested in several ways: by a marked increase in lactate dehydrogenase (LDH) and alkaline phosphatase (ALP) leakage, significant inhibition of *p*-aminohippurate (PAH) accumulation, marked depletion of intracellular ATP and reduced glutathione (GSH), and a significant reduction in pyruvate-stimulated gluconeogenesis. Co-incubation of slices with ADP or CPI and atractyloside completely blocked atractyloside-induced increase in LDH leakage, but not ALP leakage. Atractyloside-induced depletion of ATP and reduced gluconeogenesis was prevented by co-incubation with ADP or CPI. Furthermore, co-incubation of slices with STV and atractyloside, but not PRB, completely abolished atractyloside-induced depletion of ATP and decreased gluconeogenesis in the slices. Pre-incubation of slices with either ADP or CPI protected against atractyloside-induced increase in LDH leakage, reduced ATP and decreased gluconeogenesis. PAH uptake in the slices was inhibited by atractyloside and PRB in a time-dependent manner. While ADP and CPI were found to exert complete protection against atractyloside-induced toxicity irrespective of treatment schedule, STV is effective only under certain conditions, and PRB offer no protection at all. The results of this study demonstrate the usefulness of renal cortical slices as toxicology tool for evaluating and screening compounds for their potential protective effects, and are supportive of a role of adeninine nucleotide (ADP) and protease inhibitor (CPI) in protecting against atractyloside-induced cell injury.

Keywords Atractyloside toxicity · Calpain inhibitor I · ADP · Stevioside · Probenecid · Nephroprotectants Kidney slices · Renal transport

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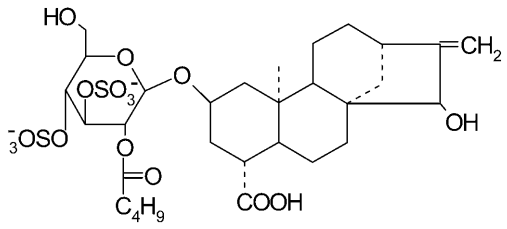
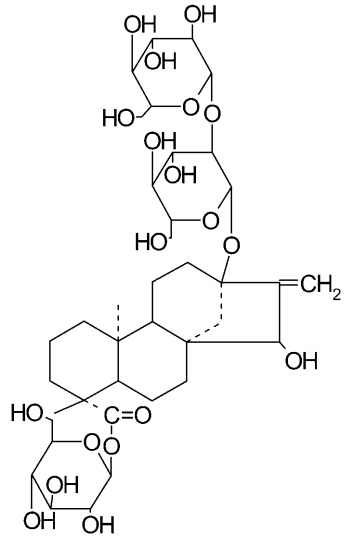
Introduction

Renal failure has been linked to the consumption of plants containing atractyloside, a diterpenoid glycoside (Fig. 1). It is well established that this compound produces an acute, mainly proximal tubule, functional impairment within hours of administration to animals (Carpenedo et al. 1974; Luciani et al. 1978) and also exhibits cell specific cytotoxicity in vitro, which was confined to the proximal tubule (Obatomi and Bach 1996). The long-term effects of chronic ingestion of sub-lethal quantities of atractyloside are also known to give rise to severe derangement of mitochondrial



Atractyloside



Stevioside

Fig. 1 Molecular structures of atractyloside and stevioside

morphology, destruction of the tubulin network within the cytoplasm and inhibition of cell division (Stewart and Steenkamp 2000). Atractyloside is also known to induce mild oxidative damage and inhibit oxidative phosphorylation, which prevents the synthesis of ATP and leads to failure of gluconeogenesis and ultimately cell death (Obatomi et al. 1998c). Consequently it is likely that more than one molecular mechanism is involved in atractyloside toxicity and that the mechanisms may vary with the cell type and tissue.

Despite its distinction as a potent nephrotoxic compound, no effective measures have been found to completely protect against the cellular damage caused by atractyloside. Therefore, development of new and effective strategies to diminish normal tissue toxicity of atractyloside is essential. A number of chemical agents used in in vitro and in vivo studies have provided some possibility of therapeutic intervention. We have recently shown that some antioxidants have protective effects on atractyloside-induced toxicity in renal cortical slices (Obatomi et al. 1998a). Other workers using pure mitochondria homogenate have also demonstrated that adenosine 5'-diphosphate (ADP), a mitochondrial energy modulator exerts some protective effect on atractyloside toxicity (Allman et al. 1967). Stevioside (Fig. 1), an atractyloside analogue, was shown to exert some protective effects on atractyloside-induced cytotoxicity in primary cell cultures and cell lines (Ishii and Bracht 1986). More recently, it was reported that calpain inhibitor I, a protease inhibitor, prevents cell death in renal cells exposed to diverse toxicants (Wang and Yuen 1994; Schnellmann and Williams 1998) and also that probenecid, an anion transport inhibitor, provides protection against atractyloside toxicity in canine kidney in vivo (Koechel and Krejci 1993). Studies undertaken to elucidate the mechanism of atractyloside-induced cell injury suggested that multiple pathways are activated or inactivated in parallel and any one of them could ultimately lead to cell death (Obatomi et al. 1998b; Obatomi and Bach 2000). It is therefore not surprising that, using different types of in vitro systems and animal models, a wide range of compounds provides "protective" measures.

The following studies investigated the protective potential of four candidate compounds (adenine nucleotide, stevioside, calpain inhibitor I and probenecid) in renal cortical slices as an extension to an earlier work (Obatomi et al. 1998a). The concentration of the various compounds used was based on previous findings (Allman et al. 1967; Ishii and Bracht 1986; Wolfgang et al. 1989; Toskulkao et al. 1994; Obatomi et al. 1998a; Schnellmann and Williams 1998). The renal cortical slice was chosen as the in vitro kidney model because it contains a population of cells that are selectively targeted by atractyloside and maintained in their normal in vivo architecture (Obatomi et al. 1998a, 1998b). The parameters selected for the present studies were based on previous experience with this in vitro system and because of their critical roles in reversible and irreversible cell damage. It was anticipated that the results from these studies would provide some insight on the relevance of these compounds as protective agents and perhaps lead to a better understanding of the mechanism of atractyloside toxicity.

Materials and methods

Chemicals and reagents

The following reagents were purchased from Sigma Chemical Co. (Poole, UK): atractyloside (sodium salt), Dulbecco's Modified Eagle's medium and Ham's Nutrient Mix F-12 (DMEM/F12), *p*-aminohippurate (PAH), probenecid (PRB), stevioside (STV), ADP and agarose. Calpain inhibitor I (CPI) was obtained from Boehringer Mannheim (East Sussex, UK). All other reagents were commercial products of the highest available grade. The DMEM/F12 medium used was oxygenated with 95% O_2/5% CO_2 and pH adjusted to 7.4 prior to use.

Animals and tissue preparation

Adult male Wistar rats, 180–210 g body weight, were supplied by Charles River (Kent, UK). Immediately after cervical dislocation, kidneys were removed and quickly placed in ice-cold rinse medium (Earles-Hepes, pH 7.4, previously gassed with 95% O_2/5%CO_2). Precision-cut renal cortical slices (approximately 200 μm thick) were prepared from cylindrical cores (8 mm) as previously

described (Obatomi et al. 1997) using the Krumdieck Tissue Slicer (Krumdieck et al. 1980) containing 0.1% agarose in phosphate-buffered saline.

Preparation of atractyloside and tested compounds

Atractyloside was dissolved in DMEM/F12 medium to give a final concentration of 2.0 mM, which was based on results of previous experiments (Obatomi et al. 1998c). ADP was similarly dissolved in DMEM/F12 medium to give a final concentration of 5.0 mM. CPI (1 mM), PRB (2.5 mM) and STV (2.5 mM) were dissolved in dimethyl sulfoxide (DMSO) and were present in the incubation media (DMEM/F12) at 1% v/v. This level of DMSO was non-toxic. The appropriate concentrations of chemical agents used were extrapolated from previously established studies in other in vitro systems (Allman et al. 1967; Ishii and Bracht 1986; Wolfgang et al. 1989; Toskulkao et al. 1994; Obatomi et al. 1998a; Schnellmann and Williams 1998).

Incubation with atractyloside and other compounds

All incubations were performed in 1-ml volumes and in a 24-well plates (Linbro, Flow Laboratories Ltd, Herts, UK) at 37°C. For pre-incubation experiments, slices were incubated for 1 h either with ADP, PRB, CPI or STV. The test compounds were removed and slices were exposed to atractyloside (2 mM) and incubated for 3 h on a rocker platform rotated at approximately 3 rpm. In co-incubation experiments, slices were incubated for 4 h with test compounds in medium containing atractyloside. In both experiments, the test compounds alone were assessed in parallel plates without atractyloside treatment.

After the incubation period, the slices were removed, rinsed twice with DMEM/F12 and assayed simultaneously for biochemical parameters. Intracellular lactate dehydrogenase (LDH) and alkaline phosphatase (ALP) leakage were determined in the incubation medium. The toxicity of atractyloside (2 mM) and the other chemical agents were determined separately in slices incubated for 3 h at 37°C on a rocker platform rotated at approximately 3 rpm.

Assay of enzyme leakage

At the end of incubation period, slices were removed and enzyme leakage determined in the incubation medium. Alkaline phosphatase (orthophosphate monoester phosphohydrolase EC 3.1.3.1) activity was assessed by measuring the conversion of *p*-nitrophenyl phosphate to *p*-nitrophenol at 400 nm (Plummer et al. 1986). Lactate dehydrogenase (L-lactate:NAD oxidoreductase EC 1.1.1.27) activity was assessed using the rate of NADH (0.2 mM) oxidation, based on the molar extinction coefficient of 6270 M/cm (Plummer et al. 1986). These measurements were made using the UV/VIS spectrophotometer (Perkin-Elmer Lambda 5 model). Percentage leakage of the enzymes from the slices into the medium was calculated and used as an index of cytotoxic damage and related to the percentage of enzyme leakage in the control.

Assessment of reduced glutathione (GSH)

The content of reduced GSH in the kidney slices was determined as previously described (Hissin and Hilf 1976). Trichloroacetic acetic acid (TCA, 0.5 ml, 0.39 M) was used to precipitate the protein in the slices and the level of GSH measured as soluble thiols (Obatomi et al. 1998c). Samples were then centrifuged at 3000 rpm for 10 min. An aliquot (75 µl) of the supernatant was removed and mixed with 2775 µl of 0.1 M phosphate/0.005 M ethylenediaminetetraacetic acid (EDTA) buffer (pH 8.0); 150 µl of *o*-phthaldehyde solution (74.6 mM) was added, mixed and incubated for 25 min at room temperature. Fluorescence was measured on LS-5L Luminescence spectrometer (Perkin Elmer) at excitation wavelength of 350 nm and emission of 420 nm. Glutathione level was calculated as nanograms per milligram slice protein before being expressed as percentage of control.

ATP levels in slices

The amount of ATP content in kidney slices was assayed by a luciferin-luciferase bioluminescence assay (Deluca and McElroy 1978). Details of the experimental procedure were as previously described (Obatomi et al. 1998c). Bioluminescence was quantified in a luminescence photometer (1251 Luminometer, LKB Wallac, Finland). ATP was determined from a standard curve and expressed as nanograms per milligram of protein which was finally presented as percentage of control.

Measurement of gluconeogenesis

The gluconeogenic capacity of the slices was assessed by measuring the conversion of pyruvate to glucose, which was measured by the glucose oxidase method as previously described (Obatomi et al. 1998c). After incubation with atractyloside or other compounds, slices were rinsed with phosphate-buffered saline and further incubated for another 1 h in glucose-free Krebs-Henseleit buffer, pH 7.4, containing 10 mM pyruvate (a gluconeogenic precursor). At the end of the incubation period, aliquots of the incubation medium containing the slices were assessed for glucose production (Roobol and Alleyne 1974). The glucose concentration in the medium was determined by the glucose oxidase and peroxidase assay according to the instructions in Sigma Technical Bulletin No. 510. Glucose levels were initially calculated as micrograms per milligram tissue but finally expressed as percentage relative to control.

Slice accumulation of *p*-aminohippurate

Two separate experiments were carried out to assess the ability to of slices to transport PAH. In the first series of experiments, following the incubation of chemical agents with atractyloside slices were removed and incubated for another 60 min in the medium, which contained 74 µM PAH and lactate (10 mM), and homogenized in 3% TCA. A 2-ml aliquot of the incubation medium was added to 3% TCA and samples were centrifuged at 3000 rpm for 5 min before assay. PAH was assessed in both medium and tissue and PAH uptake was initially expressed as the slice to medium ratio (s/m ratio), as previously described (Obatomi et al. 1998c), where s = mg PAH/g tissue; m = mg PAH/ml medium. Data were ultimately presented as percentage of the control accumulation at each time point for ease of comparison.

In a separate experiment to determine the kinetics of PAH uptake in the presence of PRB and atractyloside, atractyloside (2 mM) was incubated with PRB (2.5 mM) and/or PAH (2 mM) in an incubation medium on a shaking water bath at 37°C for up to 3 h. PAH uptake was determined at various intervals, and finally assessed and the results expressed as described above.

Protein measurement

The concentration of protein in slices and fragments was determined after solubilization in 1 N NaOH for at least 24 h. Total protein content was assessed by the Coomassie blue assay (Read and Northcote 1981) using bovine serum albumin as standard.

Data presentation and statistical evaluations

For all measurements made, results were expressed as percentage relative to the control. Data were expressed as means ± SE of at least three different experiments using four slices for each run. Comparisons were performed using Student's *t*-test (two-tailed) and are indicated in all figures as evaluation of the significance of the difference between controls and treated. P levels < 0.05 were considered to be statistically significant.

Results

Atractyloside-induced cellular injury

Baseline values for all parameters were established (Table 1). When renal cortical slices were incubated in organ culture for 3 h in the presence of atractyloside (2 mM), dramatic changes were observed in all parameters used as indicators of cellular damage (Table 1). There was a marked increase leakage of ALP and LDH, depletion of intracellular GSH and ATP, reduced gluconeogenesis and a significant alteration of PAH transport. Although all the parameters were affected, the rate of deterioration among the indicators varied.

Table 1 Atractyloside-induced cellular injury in renal cortical slices after 3 h incubation. Values represent the mean ± SE of three experiments in which four slices were used for each parameter. Slices were incubated for 3 h at 37°C in the absence (*control*) or presence of atractyloside. (*ALP* alkaline phosphatase, *LDH* lactate dehydrogenase, *ATP* adenosine 5'-triphophosate, *GSH* reduced glutathione, *GLUC* glucose, *PAH p*-aminohippurate)

Biochemical parameters	Units	Cellular process	Control[a]	Atractyloside-treated
ALP	μmol/min per mg protein	membrane permeability	132.4 ± 30.7	269.6 ± 41.8*
LDH	μmol/minper mg protein	membrane permeability	15.6 ± 1.8	59.4 ± 15.5**
ATP	ng/mg protein	cellular energy capacity	10.9 ± 2.6	4.43 ± 0.78**
GSH	ng/mg protein	oxidative status	178.0 ± 25.9	72.6 ± 21.3**
GLUC	mg/g tissue	glucose production	2.3 ± 0.24	0.7 ± 0.06**
PAH	*s*/*m* ratio	ion transport	15.6 ± 6.8	9.5 ± 2.9**

*$P < 0.05$, **$P < 0.01$ compared with the control
[a]These values were designated as 100% and used in Figs 2, 3, 4 and 5

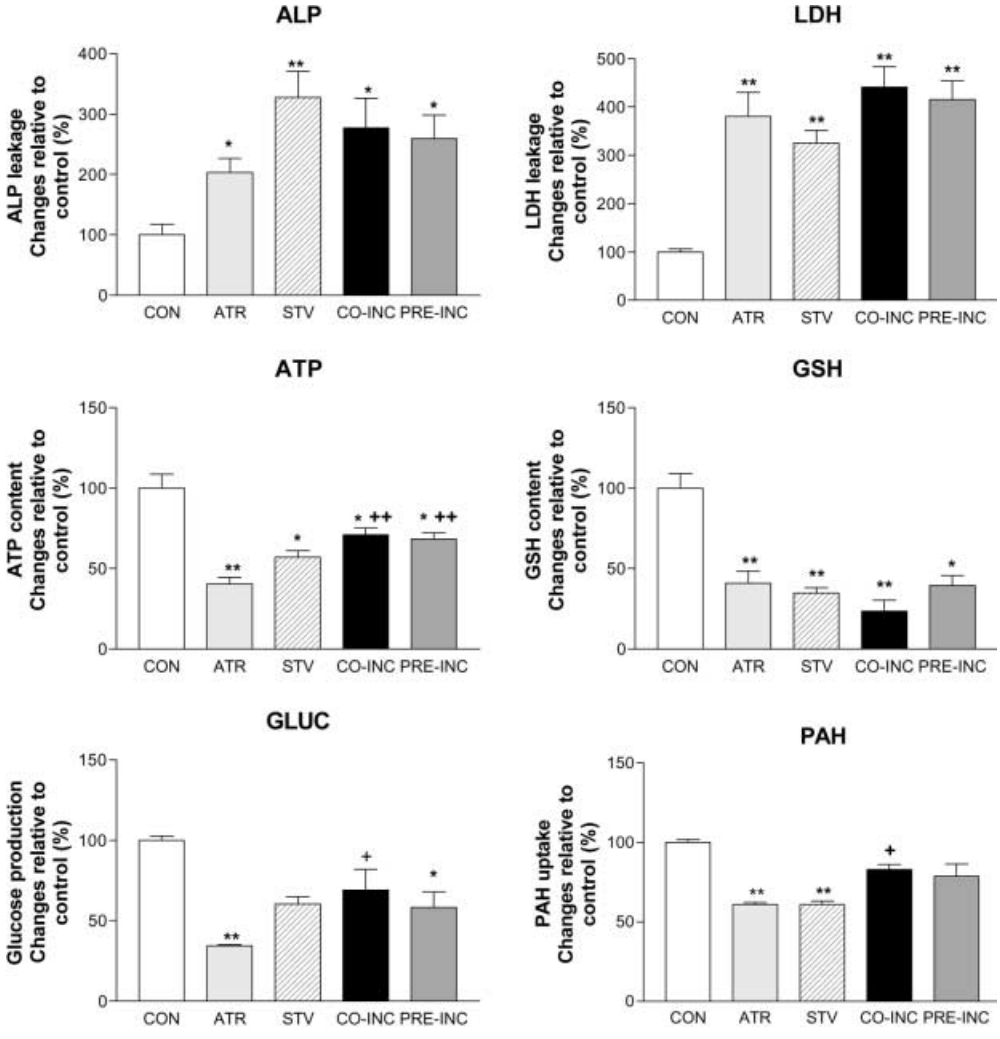


Fig. 2 The effects of co-incubation (*CO-INC*) and pre-incubation (*PRE-INC*) with stevioside (*STV*, 2.5 mM) on atractyloside-induced cellular injury in renal cortical slices. Control (*CON*) plates contain slices incubated in the absence of both STV and atractyloside (*ATR*). In addition, slices were either pre-incubated with STV for 1 h before the addition of ATR or co-incubated with STV and ATR and incubated at 37°C for 3 h. Biochemical parameters (*ALP* alkaline phosphatase, *LDH* lactate dehydrogenase, *ATP* adenosine 5'-triphophosate, *GSH* reduced glutathione, *GLUC* glucose, *PAH p*-aminohippurate) were measured and presented for all treatments. The raw data for the control was designated as 100% and all other data were related to this percentage for easy comparison. Results represent the mean ± SE of four slices from three separate experiments. *P < 0.05, **$P < 0.01$ compared with concurrent control; $^{+}P < 0.05$, $^{++}P < 0.01$ compared with ATR alone

Effects of stevioside on atractyloside-induced toxicity

In Figure 2, it is clearly apparent that renal cortical slices incubated with STV alone showed marked changes in all the parameters measured. STV exerted some direct cellular toxicity that was characterized by significant increase in LDH and ALP leakage ($P < 0.01$), marked decrease in GSH ($P < 0.01$) and ATP contents ($P < 0.05$) and reduced PAH uptake relative to control slices (Fig. 2). However, co-incubation of slices with STV and atractyloside showed some protection against atractyloside-induced depletion of ATP (by about 30%, $P < 0.05$), inhibition of gluconeogenesis (by about 35%, $P < 0.05$) and inhibition of PAH uptake (by about 25%, $P < 0.05$), while incubation of atractyloside with slices pre-incubated with STV resulted in protection against atractyloside-induced reduction in ATP (by about 30%, $P < 0.05$) (Fig. 2).

Effect of ADP on atractyloside-induced toxicity

Incubation of renal cortical slices with exogenous ADP in the absence of atractyloside caused a significant increase in GSH content ($P < 0.01$), while other parameters were similar to control (Fig. 3). Co-incubation of slices with ADP and atractyloside provide significant protection against atractyloside-induced increase in LDH leakage (by about 250%, $P < 0.01$), depletion of ATP (by about 57%, $P < 0.05$) and reduced gluconeogenesis (by about 27%, $P < 0.01$). In slices pre-incubated with ADP before addition of atractyloside, there was protection against atractyloside-induced increase in LDH leakage (by about 200%, $P < 0.05$) and against inhibition of gluconeogenesis (by about 28%, $P < 0.05$).

Effects of calpain inhibitor I on atractyloside-induced toxicity

Slices incubated with CPI alone did not cause any adverse effects on all the measured parameters (Fig. 4). Both co-incubation of slices with CPI and atractyloside and pre-incubation of slices with CPI before the addition of atractyloside had similar effects on atractyloside-induced toxicity. These effects were manifested by a significant protection against atractyloside-induced

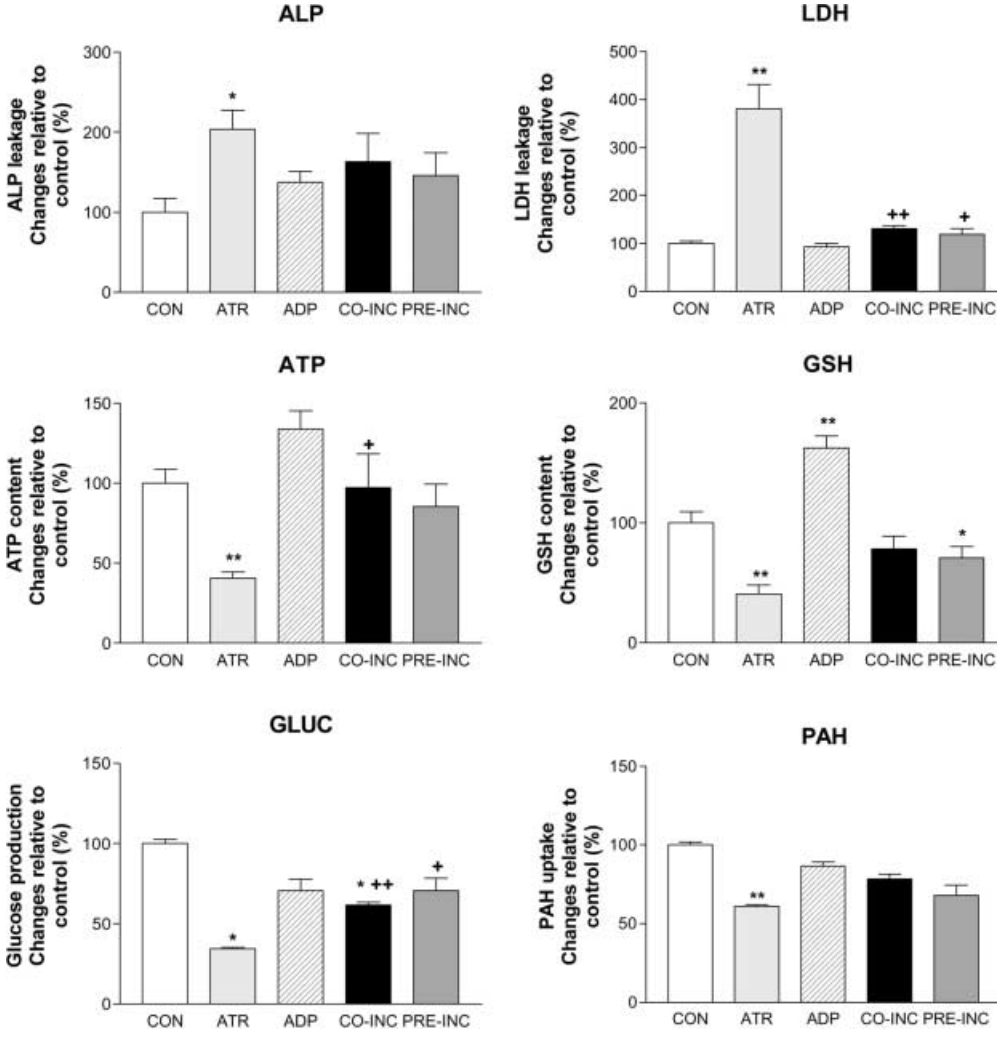


Fig. 3 The effects of co-incubation (*CO-INC*) and pre-incubation (*PRE-INC*) with adenosine 5'diphosphate (*ADP*, 5 mM) on atractyloside-induced cellular injury in renal cortical slices. Control (*CON*) plates contain slices incubated in the absence of both ADP and atractyloside (*ATR*). In addition, slices were either pre-incubated with ADP for 1 h before the addition of ATR or co-incubated with ADP and ATR and incubated at 37°C for 3 h. Biochemical parameters (*ALP* alkaline phosphatase, *LDH* lactate dehydrogenase, *ATP* adenosine 5'-triphophosate, *GSH* reduced glutathione, *GLUC* glucose, *PAH* *p*-aminohippurate) were measured and presented for all treatments. The raw data for the control was designated as 100% and all other data were related to this percentage for easy comparison. Results represent the mean ± SE of four slices from three separate experiments. $^{*}P < 0.05$, $^{**}P < 0.01$ compared with concurrent control; $^{+}P < 0.05$, $^{++}P < 0.01$, compared with ATR alone

increase in enzyme leakage (by about 150%, $P < 0.01$), depleted ATP content (by about 25%, $P < 0.05$) and reduced gluconeogenesis (by about 75%, $P < 0.01$). However, CPI did not completely protect against atractyloside-induced depletion of GSH and inhibition of PAH uptake by slices (Fig. 4).

Effects of probenecid on atractyloside-induced toxicity

Both co-incubation of slices with PRB and atractyloside and pre-incubation of slices with PRB before the addition of atractyloside have similar effect on atractyloside-induced cell injury (Fig. 5). Neither treatment protocol appeared to exert any protection against atractyloside-induced changes in any of the parameters measured. However, incubation of slices with PRB alone produced significant increase in ALP leakage and reduced gluconeogenesis (Fig. 5). In addition, co-incubation and pre-incubation of slices with PRB did not affect atractyloside-induced inhibition of gluconeogenesis.

The kinetics of PAH uptake was assessed in the presence of atractyloside alone or when incubated with PRB (Fig. 6). Accumulation of PAH was inhibited to about the same extent by PRB and atractyloside, an effect that was time-dependent. However, the inhibitory action of PRB and atractyloside on PAH uptake became significantly different from control at 90 min of incubation and beyond. Co-incubation of PRB and atractyloside significantly alter PAH kinetics with a reduction of about 40% within 30 min of incubation (Fig. 6).

Discussion

In the present study, we have demonstrated atractyloside nephrotoxicity, results of which are consistent with our previous findings (Obatomi et al. 1998a, 1998b, 1998c; Obatomi and Bach 2000). We have previously shown that the minimum period required to obtain irreversible damage in renal cortical slices following exposure to atractyloside was 3 h (Obatomi et al. 1998b; Obatomi and Bach 2000), hence the use of 3 h incubation period

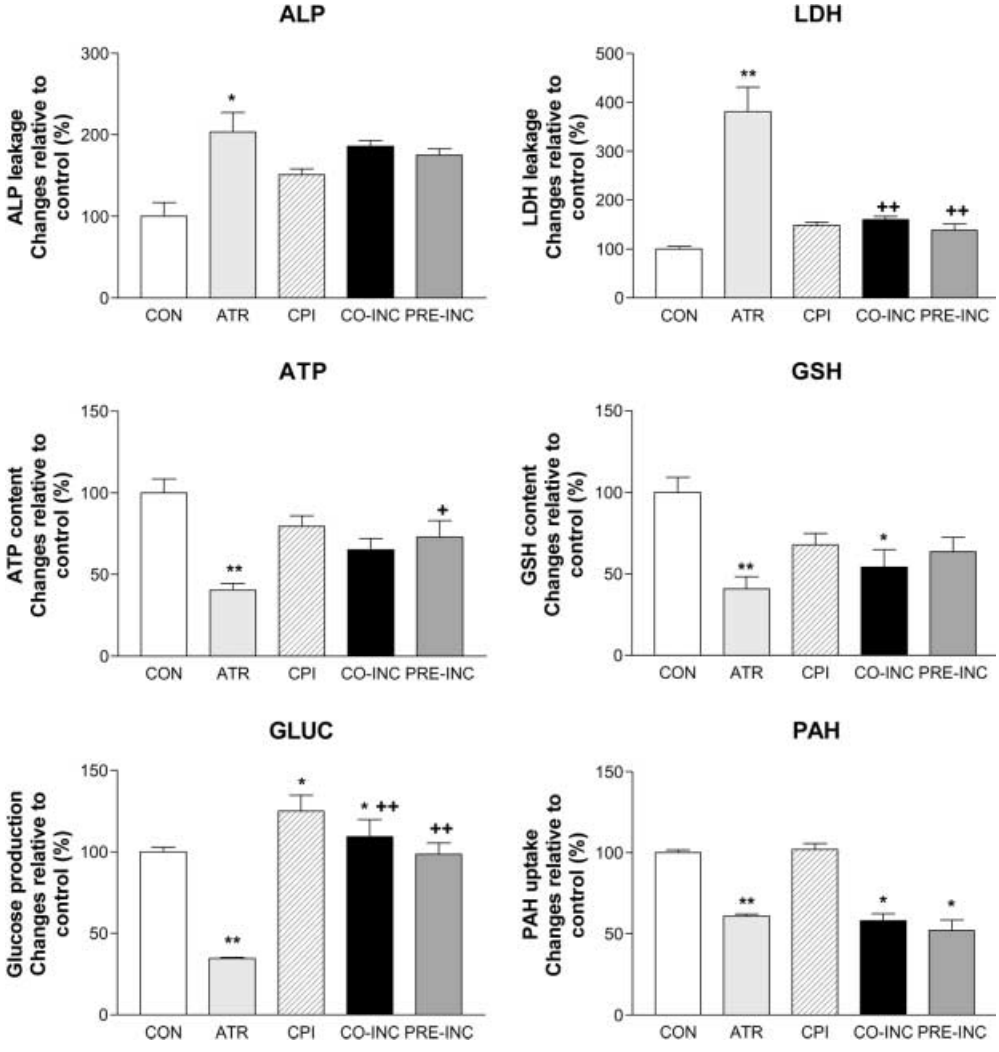


Fig. 4 The effects of co-incubation (*CO-INC*) and pre-incubation (*PRE-INC*) with calpain inhibitor 1 (*CPI,* 1 mM) on atractyloside-induced cellular injury in renal cortical slices. Control (*CON*) plates contain slices incubated in the absence of both CPI and atractyloside (*ATR*). In addition, slices were either pre-incubated with CPI for 1 h before the addition of ATR or co-incubated with CPI and ATR and incubated at 37°C for 3 h. Biochemical parameters (*ALP* alkaline phosphatase, *LDH* lactate dehydrogenase, *ATP* adenosine 5'-triphophosate, *GSH* reduced glutathione, *GLUC* glucose, *PAH p*-aminohippurate) were measured and presented for all treatments. The raw data for the control was designated as 100% and all other data were related to this percentage for easy comparison. Results represent the mean ± SE of four slices from three separate experiments. $^{*}P < 0.05$, $^{**}P < 0.01$ compared with concurrent control; $^{+}P < 0.05$; $^{++}P < 0.01$ compared with ATR alone

in the present study. This short incubation period with a high concentration of atractyloside is meant to imitate the acute in vivo situation. Our study clearly showed that co-incubation of renal cortical slices with ADP and atractyloside provided total protection against atractyloside-induced toxicity while co-incubation of slices with CPI and STV provided partial protection. In renal cortical slices pre-incubated with either CPI or ADP, but not STV or PRB, provided total protection against atractyloside-induced toxicity. Protection was adjudged to be total if all the indicators of cellular damage are returned to normal state while partial protection was based on the return to normalcy of some of the toxicity indicators used. An interesting observation was that none of the agents potentiate atractyloside-induced renal damage even though some of these compounds alone (i.e. without atractyloside) exerted some direct cellular toxicity.

The toxic effects of atractyloside have been related to its structure for which the aglycone moiety (atractyligenin) has been identified as the main component necessary to enhance toxicity (Santi 1964). However, while some structural analogues of atractyloside have been found to be equally potent, some are also weakly toxic. An example of the latter group is stevioside (STV), a glycoside obtained from *Stevia rebaudiana*, the aglycone of which is similar to atractyloside but the COOH carbonyl group located at the C4 position in atractyloside is replaced by glucose ester (see Fig. 1). STV is also an inhibitor of adenosine nucleotide translocase but it is considerably less toxic than atractyloside (Kelmer-Bracht et al. 1985). In the present study, STV showed some mild toxicity in slices but also exhibits partial protective effect on atractyloside toxicity, specifically protecting against atractyloside-induced reduction of gluconeogenesis, a result similar to that reported in hepatocytes exposed to both atractyloside and STV (Ishii and Bracht 1986). The protective ability of STV against atractyloside-induced toxicity was then ascribed to the difference in size of the two compounds. STV is presumed to compete with atractyloside in the same medium for uptake into cells although it is clearly understood that STV is unable to traverse the plasma membrane. It is possible that STV may occupy the same site of entry as atractyloside, thereby blocking atractyloside entry into cells and invariably reducing its toxic effect on those cells. To support this view is the observation that only co-incubation of STV with atractyloside provided such

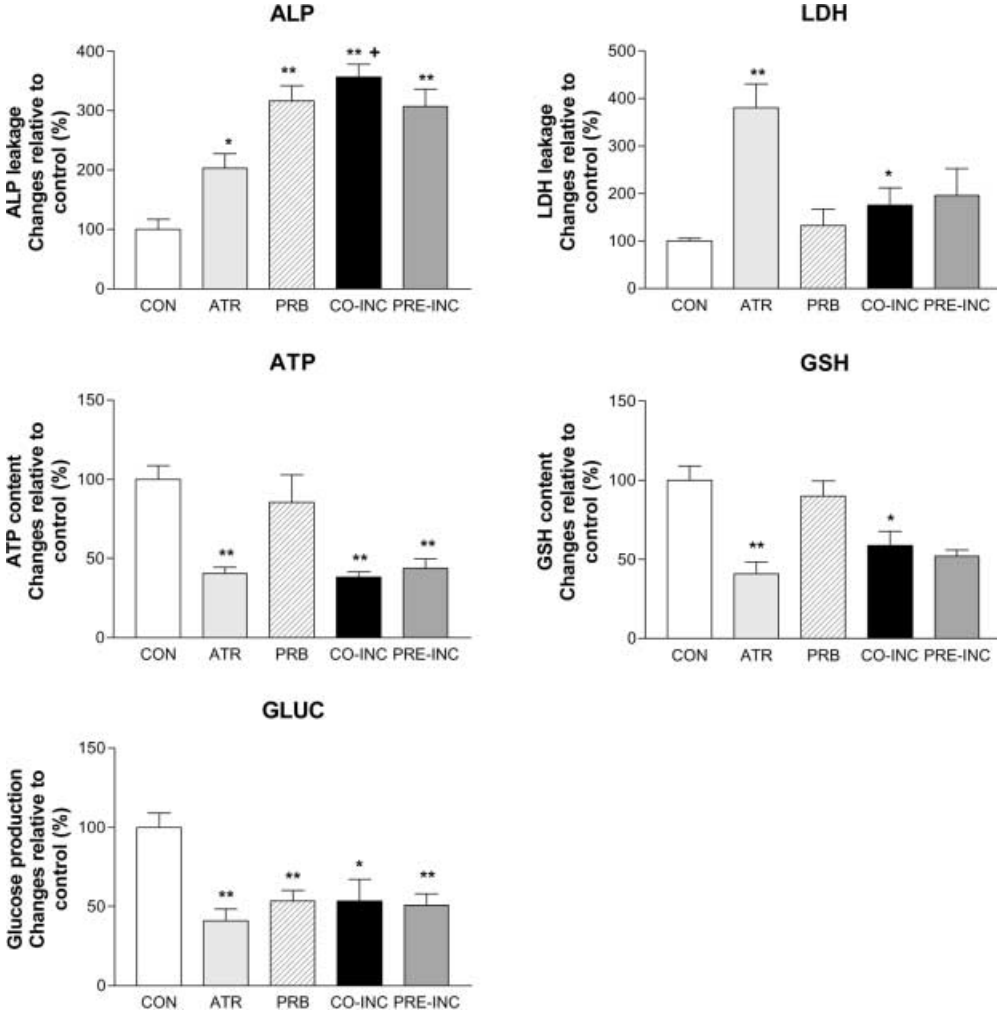


Fig. 5 The effects of co-incubation (*CO-INC*) and pre-incubation (*PRE-INC*) with probenecid (*PRB,* 2.5 mM) on atractyloside-induced cellular injury in renal cortical slices. Control (*CON*) plates contain slices incubated in the absence of both PRB or atractyloside (*ATR*). In addition, slices were either pre-incubated with PRB for 1 h before the addition of ATR or co-incubated with PRB and ATR and incubated at 37°C for 3 h. Biochemical parameters (*ALP* alkaline phosphatase, *LDH* lactate dehydrogenase, *ATP* adenosine 5'-triphophosate, *GSH* reduced glutathione, *GLUC* glucose, *PAH p*-aminohippurate) were measured and presented for all treatments. The raw data for the control was designated as 100% and all other data related to this percentage for easy comparison. Results represent the mean ±SE of four slices from three separate experiments. $^{*}P < 0.05$, $^{**}P < 0.01$ compared with concurrent control; $^{+}P < 0.05$ compared with ATR alone

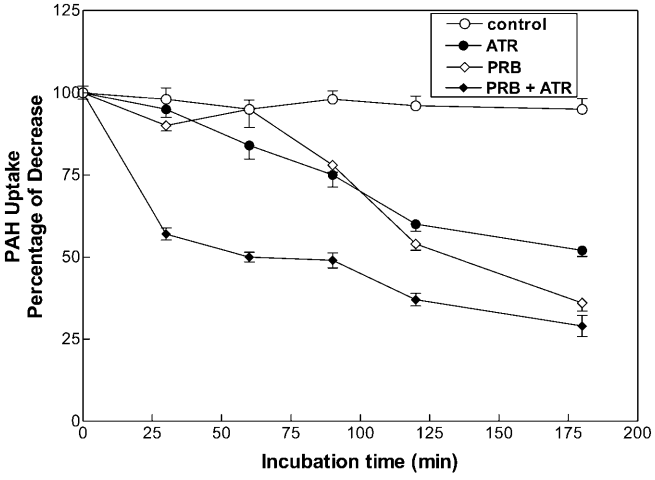


Fig. 6 Time-dependent interaction among atractyloside, probenecid (*PRB*) and *p*-aminohippurate (*PAH*) at 37°C. PAH (2 mM) was added to either atractyloside (*ATR*, 2 mM) and or PRB and incubated for up to 3 h. PAH uptake was determined over this period

protection whereas pre-incubation study could not offer such a result.

It is well established that atractyloside inhibits oxidative phosphorylation by competitively inhibiting the transport of endogenous ADP (which plays a primary role in the energy production in tissues) across the mitochondrial membrane (Bruni et al. 1962). This type of competitive inhibition was found to be reversed by addition of exogenous ADP in rat liver mitochondria (Vignais et al. 1978). In the present study, we have investigated whether such protection is reproducible in a more complex in vitro system that closely resembles the in vivo situation. While co-incubation of slices with exogenous ADP and atractyloside in the same incubation medium provides complete protection against atractyloside-induced cellular injury as assessed by all indicators of toxicity, pre-incubation of slices with ADP failed to prevent ATP depletion and LDH release. Since the extent of plasma membrane and mitochondrial damage is currently accepted as an indicator of irreversible cell damage, we can conclude that the latter treatment failed to reverse cell damage caused by atractyloside. Since total protection of atractyloside-induced toxicity was observed only when ADP was co-incubated with atractyloside, we can conclude that the mechanism involved must be competitive in nature. That is, ADP must have been preferentially taken up into slices from the incubation medium rather than atractyloside. With a reduced level of atractyloside entry into the cells, the toxic effect is reduced drastically. If this supposedly is the situation, it suggests that intracellular distribution of atractyloside plays a role in its toxicity. This viewpoint is further supported by an earlier observation that ADP competitively inhibits atractyloside (Vignais et al. 1978). This competitive type of inhibition has been linked to the resemblance of the molecular size (charge distribution) and geometry of atractyloside and ADP (Vignais et al. 1978). The polar character of the sulfate groups of atractyloside corresponds to those of the phosphate groups of ADP, the glycosidic group of atractyloside corresponds to the ribose group of ADP, and the steroid moiety of atractyloside has a geometry and hydrophobic resemblance to the purine ring system of adenine. However, this structural relationship is entirely different from that of STV, hence ADP and STV produced different effects in the presence of atractyloside.

The neutral protease calpain is one of the many intracellular proteins the activity of which depends on intracellular Ca^{2+} levels. Calpain I is one of the isoforms of calpain and is strongly expressed in the kidney. Recent data have shown that atractyloside induces the release of cathepsin B (which is identical to caspase-processing protease) from purified lysosomes (Vancompernolle et al. 1998) and this is supposed to be one of the mechanism by which atractyloside exhibits its toxicity. Calpain-inhibitors (protease inhibitors) are generally known to block the active site of calpains, the translocation of calpain proteases and thereby prevent cell death produced by toxicants. It has been demonstrated that inhibitors of calpain (CPI) protect against organ injury (McDonald et al. 2001) including renal injury induced by a variety of diverse toxicants (Schnellmann and William 1998). In the present study CPI protected against atractyloside-induced loss of pyruvate-stimulated gluconeogenesis, reduced ALP and LDH leakage, and partly reversed decreased ATP, GSH levels and PAH accumulation in the slices. In the case of CPI, both co-incubation and pre-incubation experiments provided similar results. It is therefore possible that the presence of CPI either with or without atractyloside serves to protect the cell from any adverse effect and thus, under our conditions, protects against atractyloside-induced cellular injury. This result further highlights a role for the activation of neutral proteases in atractyloside-induced nephrotoxicity and suggests CPI could be of classified as a cytoprotectant. However, the mechanism involved in this protective pathways is still not clear, although it has previously emerged that activation of calpain results in the proteolysis of several cellular proteins and enzymes, effects that are reversed by CPI as observed in this study.

The observation that PRB was effective in protecting against atractyloside-induced toxicity in vivo (Koechel and Krejci 1993), coupled with our recent findings using in vitro systems (Obatomi et al. 1998b; Obatomi and Bach 2000), led to the belief that atractyloside may be transported by the organic anion system. This hypothesis appears to be attractive when it is considered in the context of the structure of atractyloside, which has a sulfated glycoside group with an aglycone moiety (Figure 1), and is supported by earlier experiments showing that it specifically targets proximal tubular cells (Obatomi and Bach 1996). The results of the present study showed that atractyloside or PRB similarly inhibits the anion (PAH) uptake in a time-dependent fashion (Fig. 6). The combined effect of these two compounds

further reduced the kinetics of PAH. In addition, no protection was seen against atractyloside-induced toxicity, as determined by the biochemical parameters, when PRB was either co-incubated or pre-incubated with atractyloside (Fig. 5). Thus, PRB has failed to reverse the inhibition of PAH uptake caused by atractyloside in the in vitro model system that we used. The lack of protective effect of PRB would seem to suggest that the organic anion transporter is not involved. These results appear to be similar to the findings of Rankin et al. (1987), who found that the in vivo protection of PRB against a toxicant was not reproducible in vitro. One obvious interpretation that could be given to this is that, since PRB is a competitive inhibitor, it would not be expected to abolish the toxicity completely in an in vitro situation where the toxicant is not cleared from the test system. It is possible also that functional changes in organic anion transport can occur by simple competition for the same site without necessarily altering the toxicity profile. Thus atractyloside may not appear to compete with the organic anion PAH for entry into the cell as previously envisaged. In such a situation, the possible use of PRB to block renal uptake of atractyloside may not be justified.

The results presented in this paper indicate that energy depletion and cell death characterize atractyloside toxicity. We have shown that co-incubation of ADP or CPI with atractyloside offers protection at the plasma membrane, mitochondrial and cellular levels. These compounds could therefore be used strategically to block normal tissue toxicity induced by atractyloside. Furthermore, while pre-incubation of STV with atractyloside offers no protection against injury inflicted by atractyloside, co-incubation of STV with atractyloside does protect against atractyloside-induced gluconeogenesis, but not against other cellular dysfunction. On the other hand, PRB unexpectedly has no direct protective effect on atractyloside toxicity in the in vitro system used. These findings also suggest that compounds that have some structural relationships to atractyloside have the ability to modulate its toxic effect. Collectively, these experiments demonstrate the feasibility of using renal cortical slices to determine the effects of drug exposure and the different pathways involved in the protective action of some chemical agents. Ultimately, the understanding of the protective pathways of these agents could pave the way for their clinical application and thus reduce the risk of fatality that often occurs with atractyloside poisoning.

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