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ALTERATIONS IN ATP-DEPENDENT CALCIUM UPTAKE BY RAT RENAL CORTEX MICROSOMES FOLLOWING OCHRATOXIN A ADMINISTRATION *IN VIVO* OR ADDITION *IN VITRO*

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Abstract—A disruption of calcium homeostasis, leading to a sustained increase in cytosolic calcium levels, has been associated with cytotoxicity in response to a variety of agents in different cell types. We have observed that administration of a single high dose or multiple lower doses of the carcinogenic nephrotoxin ochratoxin A (OTA) to rats resulted in an increase of the renal cortex endoplasmic reticulum ATP-dependent calcium pump activity. The increase was very rapid, being evident within 10 min of OTA administration and remained elevated for at least 6 hr thereafter. The increase in calcium pump activity was inconsistent with previous observations that OTA enhances lipid peroxidation (ethane exhalation) *in vivo*, a condition known to inhibit the calcium pump. However, no evidence of enhanced lipid peroxidation was observed in the renal cortex since levels of malondialdehyde and a variety of antioxidant enzymes including catalase, DT-diaphorase, superoxide dismutase, glutathione peroxidase, glutathione reductase and glutathione *S*-transferase were either unaltered or reduced. In *in vitro* studies, addition of OTA to cortex microsomes during calcium uptake inhibited the uptake process although the effect was reversible. Preincubation of microsomes with NADPH had a profound inhibitory effect on calcium uptake but inclusion of OTA was able to reverse the inhibition. Changes in the rates of microsomal calcium uptake correlated with changes in the steady-state levels of the phosphorylated Mg^{2+}/Ca^{2+}-ATPase intermediate, suggesting that *in vivo/in vitro* conditions were affecting the rate of enzyme phosphorylation.

Ochratoxin A (OTA)†, a mycotoxin consisting of a 5′-chlorinated 3,4-dihydro-3-methylisocoumarin moiety linked by an amide bond to L-phenylalanine, is produced by several species of *Aspergillus* and *Penecillum*. The natural occurrence of OTA in food and feed is widespread [1], and it has been detected in blood [2] and tissues [3] of slaughter pigs as well as in blood of humans [4, 5]. Ochtratoxin A is nephrotoxic to all single-stomached animals and is linked to outbreaks of nephropathy in pigs and chickens [1, 6]. Ochratoxin A has been shown to be a renal carcinogen in mice [7] and rats [8] and is suspected of being the main etiologic agent responsible for endemic Balkan nephropathy and associated urinary tract tumors in humans [9]. Alterations in a variety of biochemical and immunological parameters have been observed following OTA administration [10–13]; however, the mechanism of its toxicity remains unknown.

Intracellular free calcium mediates a variety of physiological and biochemical processes and its compartmentation within the cell is governed by several transport systems which operate in a highly regulated fashion. Loss of normal calcium homeostasis, leading to a sustained increase in cytosolic calcium levels, appears to be an early and critical event in the development of toxic injury by diverse chemicals in a variety of cell types [14, 15]. The endoplasmic reticulum (microsomal) ATP-dependent calcium pump is an important factor in the regulation of cytosolic calcium homeostasis [16, 17]. This microsomal calcium pump is very sensitive to oxidative damage, and several toxic agents, which can either directly (e.g. hydroperoxides) or indirectly (e.g. carbon tetrachloride) cause oxidant damage, are also known to decrease the activity of the microsomal calcium pump [14, 18–21]. We have shown previously that addition of OTA to rat liver microsomes stimulates lipid peroxidation and that this results in a drastic inhibition of ATP-dependent calcium uptake [22]. Administration of OTA to rats also lowers the rate of ATP-dependent calcium uptake by liver microsomes when measured *ex vivo* [22]. In view of the fact that OTA is primarily a renal toxin, we have presently examined the effects of OTA administration *in vivo* or addition *in vitro* on the ATP-dependent calcium uptake by renal cortex microsomes as well as the levels of renal lipid peroxides [malondialdehyde (MDA)] and a variety of oxidative enzymes.

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† Abbreviations: CDNB, 1-chloro-2,4-dinitrobenzene; DCPIP, 2,6-dichlorophenol indophenol; DMSO, dimethyl sulfoxide; GSH, reduced glutathione; GSSG, oxidized glutathione; MDA, malondialdehyde; OTA, ochratoxin A; and TBA, 2-thiobarbituric acid.

MATERIALS AND METHODS

Chemicals. ATP, 1-butanol, dimethyl sulfoxide (DMSO), EDTA, NADPH, OTA, 2-thiobarbituric acid (TBA), xanthine, 2,6-dichlorophenol indophenol (DCPIP), Tween 20, 1-chloro-2,4-dinitrobenzene (CDNB), dicoumarol, reduced glu-

tathione (GSH), oxidized glutathione (GSSG), ferricytochrome *c* and xanthine oxidase were purchased from the Sigma Chemical Co. (St. Louis, MO). CDNB was crystallized from ethanol before use. $^{45}CaCl_2$ (10 mCi/mg) and [γ-^{32}P]ATP (5000 Ci/mmol) were obtained from Du Pont Canada Inc. (Montreal, Quebec) and Amersham Canada Ltd. (Oakville, Ontario), respectively. All other chemicals were of the highest grade commercially available.

Treatment of animals and preparation of microsomes. Male Sprague–Dawley rats (225–275 g) were obtained from Charles River Canada and were maintained on standard laboratory chow and water *ad lib*. OTA was administered i.p. in 0.1 M sodium bicarbonate (~0.5 mL/rat). Control rats received an equivalent amount of sodium bicarbonate solution. Subsequent to dosing, rats were killed at timed intervals as indicated in the legends to the figures and tables. The kidneys were removed, and microsomes and 105,000 *g* supernatant were prepared from the cortices of individual rats as described earlier [23]. Protein was determined by the method of Lowry et al. [24].

In vitro *preincubations*. Prior to measuring calcium uptake, cortex microsomes from untreated rats were preincubated with NADPH (0.75 mM) in the presence or absence of OTA. For OTA concentration studies, cortex microsomes (1.0 mg protein/mL) were preincubated in 0.1 M potassium phosphate buffer (pH 7.4) for 10 min at 37° with a range of OTA concentrations (0 to 1.0 mM; added in a maximum volume of 5 μL of DMSO/mL of incubation) in the presence or absence of NADPH (0.75 mM). For the time course, cortex microsomes (1.0 mg protein/mL) were incubated as above with OTA (1.0 mM) but for various lengths of time (2–60 min). At the end of the incubation, the mixtures were immediately cooled on ice and centrifuged at 105,000 *g* for 30 min at 4° to recover the microsomes. The microsomal pellets obtained were resuspended in imidazole-histidine buffer (30 mM imidazole, 30 mM histidine, 100 mM KCl; adjusted to pH 6.8 with imidazole) to a concentration of 0.5 mg protein/mL and assayed for calcium uptake as described below.

Measurement of calcium uptake. Calcium uptake activity in cortex microsomes from control or OTA-treated rats or in *in vitro* pretreated microsomes was measured by the method of Moore [25]. Briefly, microsomes (0.1 to 0.15 mg protein/mL) were added to ice-cold imidazole-histidine buffer (pH 6.8) containing 5 mM $MgCl_2$, 5 mM ATP, 5 mM ammonium oxalate, 20 μM $CaCl_2$ and 0.1 μCi/mL $^{45}CaCl_2$. A 0.5-mL aliquot was filtered immediately with the aid of a vacuum apparatus on pre-wetted glass microfibre filters (Whatman, type 934-AH; diameter 24 mm; pore size 0.2 μm) as zero time. The reactions were then started by placing the tubes in a 37° shaking water-bath and at the indicated time intervals, 0.5-mL aliquots were taken and filtered as described above. After rapid washing with 10 mL of cold buffer, individual filters were placed in separate scintillation vials and air dried. Bound radioactivity was monitored in a Beckman LS-1801 liquid scintillation counter after addition of 5 mL of scintillation fluid (ScintiVerse II, Fisher Chemical Co.). ATP dependence of calcium uptake was shown by excluding it from the incubation medium.

Measurement of ATP levels. For measurement of ATP levels, microsomes were preincubated with NADPH in the presence of OTA (1 mM) for 10 min, then centrifuged, and the resuspended microsomes incubated for calcium uptake as described above except that $^{45}Ca^{2+}$ was omitted. At timed intervals, 0.5-mL aliquots were withdrawn and mixed immediately with 2 mL of 0.6 M perchloric acid to stop the reaction. The samples were brought to a pH of ~6.5 by addition of 4 mL of 0.1 M phosphate buffer (pH 7.5) and 0.3 mL of 30% KOH. The precipitated potassium perchlorate was removed by filtration through a 0.45 μm nylon filter (Cameo II, MSI, Westboro, MA), and ATP levels were measured in the supernatant by HPLC as described by Stocchi *et al.* [26].

Measurement of calcium backflux. Microsomes (3 mg/mL) were passively loaded with $^{45}Ca^{2+}$ by incubation at 0° for 2 hr in 2 mL of buffer (pH 7.5) consisting of 100 mM KCl, 5 mM $MgCl_2$, 20 mM HEPES and 1 mM $^{45}CaCl_2$ (0.7 μCi) [27]. Efflux of $^{45}Ca^{2+}$ was measured as described by Tsokos-Kuhn *et al.* [28] and was initiated by diluting 100 μL of the above mixture (in triplicate) with 2.9 mL of ice-cold buffer containing 100 mM KCl, 5 mM $MgCl_2$, 20 mM HEPES, 2 mM ethyleneglycolbis(aminoethylether)-tetra-acetate (EGTA) and various concentrations of OTA (0–1 mM). At timed intervals, 0.4-mL aliquots were withdrawn and passive backflux of calcium was monitored by measuring the microsomal calcium content as described under "Measurement of calcium uptake".

Measurement of lipid peroxidation. Lipid peroxidation in the samples was monitored by measuring the level of MDA as described by Uchiyama and Mihara [29]. Briefly, 0.5 mL of the cortex homogenate was mixed with 3 mL of 1% phosphoric acid to keep the pH of the medium at about 2.0. One milliliter of 0.6% aqueous TBA was added and the mixture was heated to 100° for 45 min. After cooling, 4 mL of 1-butanol was added and the mixture was shaken vigorously. The butanol phase was separated by centrifugation, and its absorbance at 535 and 520 nm was measured. The difference in absorbance at these two wavelengths was taken to calculate the MDA–TBA value using tetramethoxypropane as the external standard.

Phosphorylation of microsomal vesicles. Phosphoprotein levels were measured by labeling microsomes with [γ-^{32}P]ATP, followed by electrophoretic analysis on sodium dodecyl sulfate (SDS) polyacrylamide gels [30]. Briefly, phosphorylation reactions were carried out at 0° in a volume of 0.1 mL and contained 37 mM HEPES (pH 6.8), 0.1 M KCl, 50 μM $CaCl_2$, 2.5 mM sodium azide, ~5 nM [γ-^{32}P]ATP (5000 Ci/mmol) and 30 μg microsomal protein. The reaction was stopped after 1 min by addition of 0.1 mL of a solution containing 8% SDS (w/v), 10% 2-mercaptomethanol (v/v) and 188 mM Tris–HCl (pH 6.8). Samples were incubated for 15 min at 37° and then mixed with 50 μL of 100% sucrose. SDS-PAGE was carried out by the method of Laemmli [31] as described by Spamer *et al.* [30]. Proteins were stained with Commassie Brilliant Blue

R-250 to distinguish the bands. Destained gels were dried by heat under vacuum and exposed to Kodak X-Omat RO film. The protein band corresponding to the ^{32}P-phosphorylated protein ($M_r \sim 116{,}000$) was then cut and solubilized with 0.25 mL of 30% peroxide at 50°, and bound radioactivity was quantitated by liquid scintillation counting.

Measurement of enzyme activities. Catalase activity of cytosol was measured according to Aebi [32] by following the decrease in absorbance of H_2O_2 at 240 nm ($E_{240} = 34.9\,M^{-1}\,cm^{-1}$). Superoxide dismutase (SOD) activity was measured by a modification of the method described by McCord and Fridovich [33] and is based on the ability of SOD present in cytosol to inhibit the xanthine-xanthine oxidase-dependent reduction of ferricytochrome *c* to ferrocytochrome *c*. One unit of SOD is defined as that amount of enzyme required to inhibit cytochrome *c* reduction by 50%. Glutathione peroxidase activity of cytosol was measured as described by Lawrence and Burk [34]. Briefly, this involved following the oxidation of NADPH ($E_{340} = 6.22\,mM^{-1}\,cm^{-1}$) at 37° in 50 mM potassium phosphate buffer (pH 7.0) containing sodium azide (1 mM), GSH (1 mM), EDTA (1 mM), H_2O_2 (0.25 mM), glutathione reductase (1 U/mL) and NADPH (0.2 mM). Glutathione reductase was assayed spectrophotometrically according to Flohé and Gunzler [35] by measuring the rate of oxidation of NADPH ($E_{340} = 6.22\,mM^{-1}\,cm^{-1}$) in a 1-mL cuvette containing 50 mM potassium phosphate buffer (pH 7.6), 0.1% bovine serum albumin, GSSG (3.3 mM) and NADPH (0.1 mM). GSH *S*-transferase activity was measured by following the change in absorbance of CDNB ($E_{340} = 9.6\,mM^{-1}\,cm^{-1}$) in 0.1 M phosphate buffer (pH 6.5) containing GSH (1 mM), CDNB (1 mM) and cytosol [36]. DT-Diaphorase activity of cytosol was measured by following the reduction of DCPIP ($E_{500} = 21.0\,mM^{-1}\,cm^{-1}$) at 25° in 25 mM Tris–HCl buffer (pH 7.5) containing 0.07% bovine serum albumin, 0.01% Tween 20, flavin adenine dinucleotide (5 μM) DCPIP (40 μM), NADPH (0.2 mM) and dicoumarol (10 μM) [37].

Statistical analyses. Data were analyzed for significant differences by Student's *t*-test or two-way analysis of variance (ANOVA) as appropriate with a level of significance being taken as $P < 0.05$.

RESULTS

Administration of a single high dose of OTA (10 mg/kg) significantly increased the rate of ATP-dependent calcium uptake by renal cortex microsomes. Figure 1 shows the rates of calcium uptake by cortex microsomes isolated from rats that had been treated 10 min, 2 hr, 6 hr and 12 hr earlier with OTA or vehicle. The rate of calcium uptake was linear for about 20 min but continued to increase throughout the 40-min incubation. A significant increase in the rate of calcium uptake was evident as early as 10 min after OTA dosing (Fig. 1A) and a maximum increase of ~80% was observed at 2 hr (Fig. 1B). However, by 12 hr there was no significant difference in the rates of calcium uptake between control and OTA-treated groups (Fig. 1D). Single lower doses of OTA led to the correspondingly lower increases in the rate of calcium uptake while no increase over control was observed at or below a dose of 2 mg/kg OTA (data not shown).

A mixed response was observed on multiple dosing of rats with OTA. Lower doses of OTA elevated the rate of ATP-dependent calcium uptake while higher doses depressed it. Daily dosing with 1–2 mg/kg OTA for 4 days increased the rate of calcium uptake with a maximum increase of ~45% being observed at a dose of 1 mg/kg (Fig. 2, B and C). An increase in calcium uptake was also observed at 0.5 mg/kg OTA but this was not significant (Fig.2A). In contrast, daily dosing with 2 mg/kg OTA for 8 days or 4 mg/kg OTA for 4 days resulted in a 40–50% inhibition of the calcium uptake activity (Fig. 3). Previously, Berndt and Hayes [10] showed that daily dosing of rats with 0.75 and 2 mg/kg for 5 days impaired renal function as judged by inhibition of anion transport and persistent urinary hypoosmolarity coupled with excessive glucose and protein excretion.

In *in vitro* studies, the presence of OTA in the incubation mixture during calcium uptake strongly inhibited the uptake reaction. Thus, the presence of 0.25, 0.5 or 1 mM OTA inhibited calcium uptake by 65, 77 or 99% (Fig. 4). This inhibitory effect of OTA was both rapid and (at least partially) reversible. The effect was rapid because addition of OTA (0.5 mM) 5 or 10 min after calcium uptake was initiated resulted in an inhibition of calcium uptake similar to that observed if OTA had been present at the start of the incubation (Fig. 5). The inhibitory effect appeared to be reversible because, in comparison to direct addition (Fig. 4), preincubation of microsomes with 0.25, 0.5 or 1 mM OTA for 10 min followed by centrifugation, led to only a 16, 36 or 55% inhibition of calcium uptake by the recovered microsomes (Fig. 6).

In an effort to determine if mixed-function oxidase-dependent metabolism of OTA altered its effect on calcium uptake, NADPH was also included in the incubation medium. Preincubation of microsomes with NADPH strongly suppressed (by ~90%) their ability to sequester calcium. However, inclusion of OTA during preincubation with NADPH resulted in a concentration-dependent reversal of this inhibitory effect (Fig. 6). Thus, the presence of 1 mM OTA "increased" the rate of calcium uptake by NADPH-preincubated microsomes 5- to 10-fold to the value observed in the presence of NADPH alone.

The microsomal calcium pump is known to be very sensitive to oxidative damage including lipid peroxidation [14, 18–21]. We therefore measured MDA levels in parallel with calcium uptake in microsomal incubations carried out in the presence of NADPH alone or NADPH along with various concentrations of OTA. The results indicate that the observed concentration-dependent reversal of NADPH inhibition of calcium uptake by OTA correlates well with the decrease in lipid peroxide levels as measured by MDA formation (Fig. 7).

Addition of OTA (up to 1 mM) to microsomes loaded with $^{45}Ca^{2+}$ did not alter the rate of calcium backflux, indicating that increased backflux was not

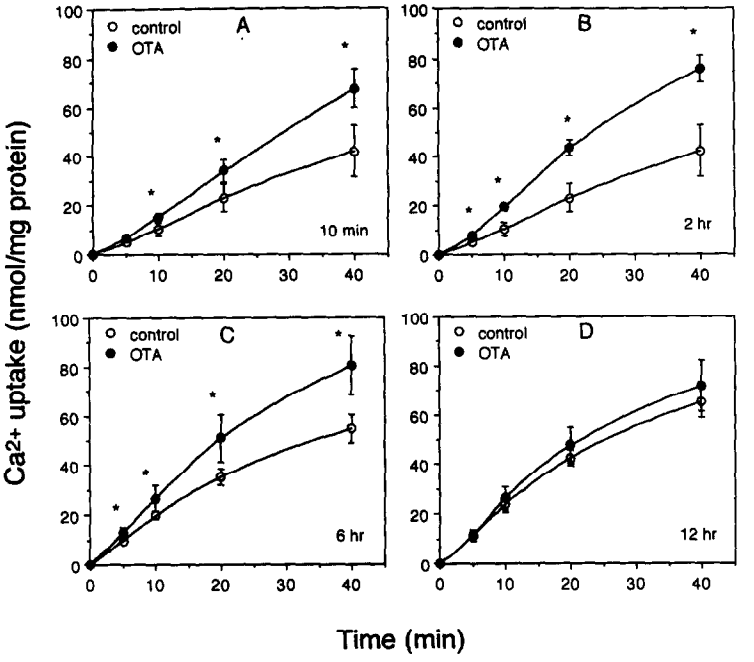

Fig. 1. Calcium uptake by renal cortex microsomes isolated from rats treated once with OTA (10 mg/kg) or vehicle (bicarbonate). Calcium uptake measurements were carried out in imidazole-histidine buffer (pH 6.8) containing 0.1 to 0.15 mg/mL microsomal protein, 5 mM $MgCl_2$, 5 mM ATP, 5 mM ammonium oxalate, 20 μM $CaCl_2$ and 0.1 μCi $^{45}CaCl_2$. At the indicated times, 0.5-mL aliquots were filtered through glass microfibre filters and washed, and bound radioactivity was determined as an index of calcium uptake by microsomes. Details are as described in Materials and Methods. Microsomes from individual rats were assayed twice, each time in triplicate. Values are means ±SD from 4–6 individual rats per time point. Key: (*) denotes a significant difference ($P < 0.01$) from control.

responsible for decreased calcium uptake in the presence of OTA (Fig. 8). Similarly, microsomes isolated from OTA-treated and control rats, when loaded with $^{45}Ca^{2+}$, showed similar rates of calcium backflux suggesting that decreased calcium backflux is not responsible for the increased rate of calcium uptake observed subsequent to OTA administration (data not shown). Likewise, differences in the rate of ATP consumption cannot account for the altered rates of calcium uptake observed after OTA administration *in vivo* or OTA/NADPH addition *in vitro* since sufficient ATP was left at the end of the incubation (40 min) so as not to be limiting (Fig. 9).

In the presence of calcium, addition of [γ-^{32}P]-ATP to liver microsomes is followed by rapid formation of the phosphorylated enzyme intermediate [30]. Our results show that alterations in the rate of calcium uptake brought about by *in vivo*/*in vitro* treatments correlated with the levels of the phosphoenzyme intermediate. Thus, OTA administration which led to an increased rate of ATP-dependent microsomal calcium uptake also showed a higher level of the phosphoenzyme intermediate as compared to control microsomes (Table 1). Conversely, the protective effect of OTA on the NADPH-dependent inhibition of calcium uptake was accompanied by a simultaneous increase in the level of the phosphoenzyme intermediate (Table 1). In the presence of 1 mM OTA there was no significant difference in phosphoenzyme levels between +NADPH and −NADPH (Table 1). This correlated well with the lack of difference in calcium uptake between +NADPH and −NADPH in the presence of 1 mM OTA (Fig. 6).

The occurrence of oxidative stress *in vivo* often results in increased levels of a variety of antioxidant enzymes [38]. We therefore measured the levels of a variety of cytosolic enzymes subsequent to dosing of rats with various concentrations of OTA. The following activities (per minute per milligram protein) were observed in renal cytosol from control rats: catalase, 55.5 ± 0.4 μmol; GSH peroxidase, 229.1 ± 29.4 nmol H_2O_2 reduced; GSH reductase, 194.4 ± 25.7 nmol GSSG reduced; GSH *S*-transferase, 445.3 ± 31.0 nmol CDNB conjugated; superoxide dismutase, 7.8 ± 1.2 U and DT-diaphorase, 85.4 ± 8.3 nmol DCPIP reduced. There were no significant changes in the levels of any of these activities in cortex supernatants of rats pretreated daily for 4 days with 0.5, 1 or 2 mg OTA/kg or in cytosol isolated from rats pretreated 10 min to 6 hr earlier with 10 mg/kg OTA (data not shown). Also, there was no significant change in MDA levels in cortex homogenates isolated from rats pretreated with 0.5 to 4 mg OTA daily for 4 days (Table 2). However, MDA levels were reduced significantly in

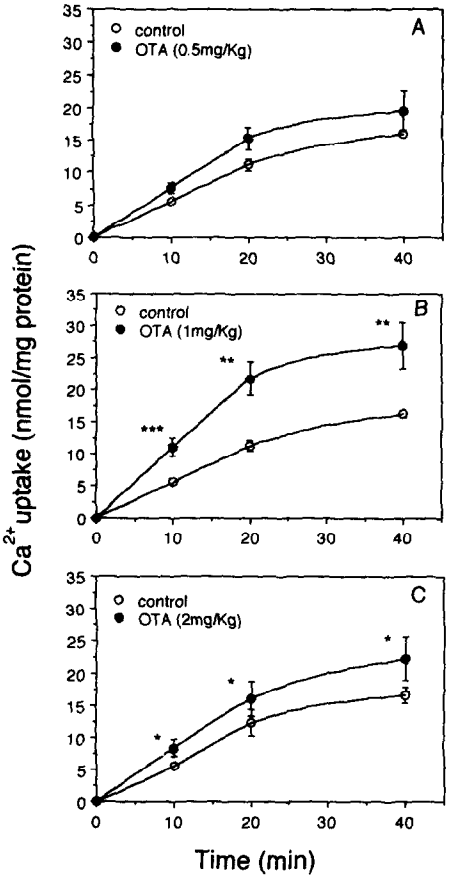

Fig. 2. Calcium uptake by renal cortex microsomes isolated from rats that received four daily doses of OTA. Rats were killed 24 hr after the last dose of OTA. Calcium uptake was measured as described in the legend of Fig. 1. Values are means ±SD from 4 individual rats per treatment group. Key: (*) denotes a significant difference ($P < 0.05$) from control; (**) denotes a significant difference ($P < 0.01$) from control; and (***) denotes a significant difference ($P < 0.001$) from control.

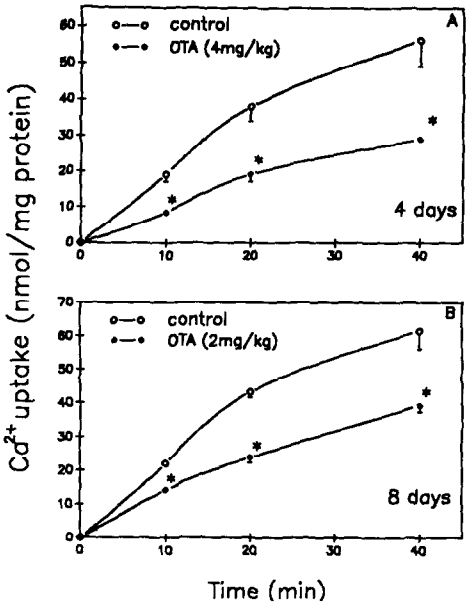

Fig. 3. Calcium uptake by renal cortex microsomes isolated from rats that received OTA daily in doses of 4.0 mg/kg for 4 days (A) or 2.0 mg/kg for 8 days (B). Rats were killed 24 hr after the final dose of OTA. Calcium uptake was measured as described in the legend of Fig. 1. Values are means ± SD from 5 individual rats per treatment group. Key: (*) denotes a significant difference ($P < 0.0001$) from control.

whole cortex homogenates prepared from rats treated 30 min to 2 hr earlier with 5 mg/kg OTA (Table 2).

DISCUSSION

Numerous chemicals of diverse structure which cause cell toxicity are also known to perturb intracellular calcium levels. This has led to the suggestion [14] that an alteration in calcium homeostasis leading to a sustained increase in intracellular calcium levels may be a common mechanism by which a variety of toxicants exert their deleterious effects. The cytosolic free calcium concentration in mammalian cells is usually quite low (~0.1 μM) compared with the concentration of free calcium in extracellular fluids (~1.3 mM). In renal tubular cells, the passive influx of calcium at the brush border, driven by its electrochemical gradient, is normally balanced by active calcium efflux at the basolateral membrane by Ca^{2+}-ATPase(s) and possibly by a Na^+/Ca^{2+} antiporter [39]. In addition, cytosolic calcium concentration is controlled by active sequestration into intracellular stores, which include mitochondria and endoplasmic reticulum, and by calcium binding to intracellular

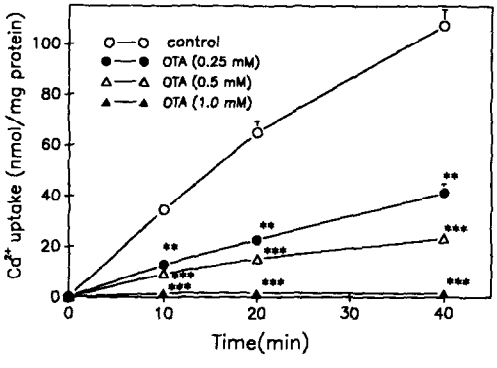

Fig. 4. Inhibitory effect of OTA on calcium uptake by renal cortex microsomes. Calcium uptake measurements were carried out as described under Fig. 1 but in the presence of various concentrations of OTA. Values are means ± SD of two experiments, each performed in triplicate. Key: (**) denotes a significant difference ($P < 0.01$) from control; and (***) denotes a significant difference ($P < 0.001$) from control. A significant difference ($P < 0.05$) was also noted between 0.25 mM OTA and 1 mM OTA and between 0.5 mM OTA and 1 mM OTA at all time points.

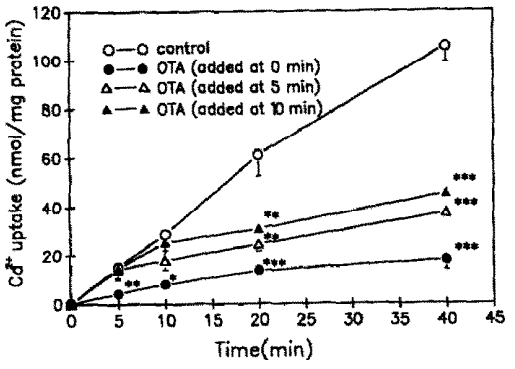

Fig. 5. Effect of delayed addition of OTA on calcium uptake by cortex microsomes. Calcium uptake was carried out as in Fig. 4 except that OTA was added at 0, 5 or 10 min after the start of the reaction. Values are means ± SD of two experiments, each performed in triplicate. Key: (*) denotes a significant difference ($P < 0.05$) from control; (**) denotes a significant difference ($P < 0.01$) from control; and (***) denotes a significant difference ($P < 0.001$) from control.

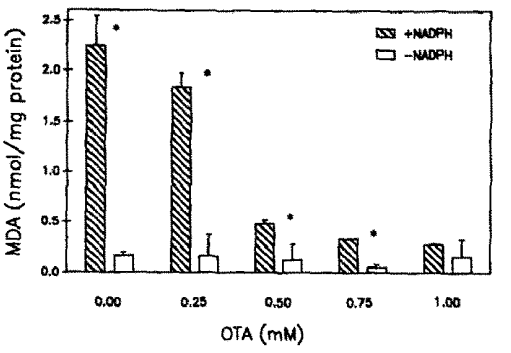

Fig. 7. Inhibition of NADPH-dependent lipid peroxidation by OTA. Microsomes were preincubated with various concentrations of OTA ± NADPH as described under Fig. 6. Lipid peroxidation was measured in the recovered microsomes by determining the levels of MDA as described in Materials and Methods. Values are means ± SD of two experiments, each performed in triplicate. Key: (*) denotes a significant difference ($P < 0.005$) between +NADPH and −NADPH at the indicated OTA concentrations. The difference in the group means (+NADPH) was also highly significant ($P < 0.0001$).

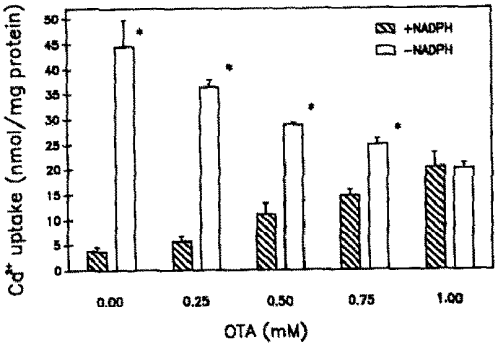

Fig. 6. Inhibition of calcium uptake by preincubation of microsomes with NADPH and its reversal by OTA. Cortex microsomes were preincubated for 10 min with the indicated concentrations of OTA in the presence or absence of NADPH. Subsequent to centrifugation, calcium uptake by the recovered microsomes was determined as described in the legend of Fig. 1. Values are means ± SD of two experiments, each performed in triplicate. Key: (*) denotes a significant difference ($P < 0.0001$) between +NADPH and −NADPH at the indicated OTA concentrations. The difference between the group means (+NADPH and −NADPH) was also highly significant ($P < 0.0001$).

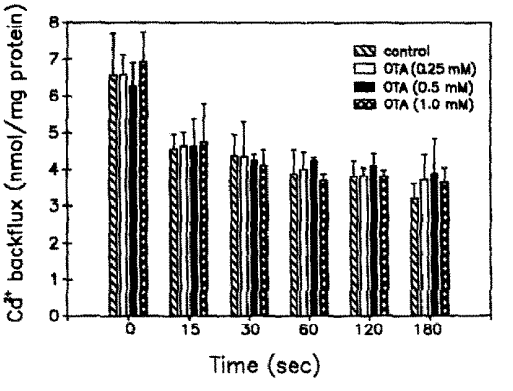

Fig. 8. Effect of OTA addition on rates of calcium backflux. Microsomes passively loaded with $^{45}Ca^{2+}$ were incubated in KCl–$MgCl_2$–HEPES–EGTA buffer (pH 7.5) in the presence of various concentrations of OTA. At timed intervals, 0.5-mL aliquots were filtered and washed, and bound radioactivity was determined as described in Materials and Methods. Values are means ± SD of two experiments, each performed in triplicate.

proteins. Mitochondria have a high capacity but a low affinity for calcium uptake thus preventing them from lowering the calcium below 10^{-6} M. In contrast, the endoplasmic reticulum has a high-affinity, low-capacity [40] or high capacity [41] Ca^{2+}-ATPase and is believed to be responsible for maintaining cytosolic calcium in the proper range [39].

Berndt *et al.* [42] first showed that calcium uptake by renal cortex slices was enhanced substantially within 5 min of OTA addition. We have shown that OTA inhibits, in a dose-dependent manner, both state 3 respiration and calcium uptake by renal cortex mitochondria.* This may lead to an increase in cytosolic calcium levels, and the increase in microsomal calcium uptake activity observed subsequent to *in vivo* OTA dosing may help to restore calcium homeostasis. Moreover, the increase

* Khan S and Rahimtula AD, manuscript submitted for publication.

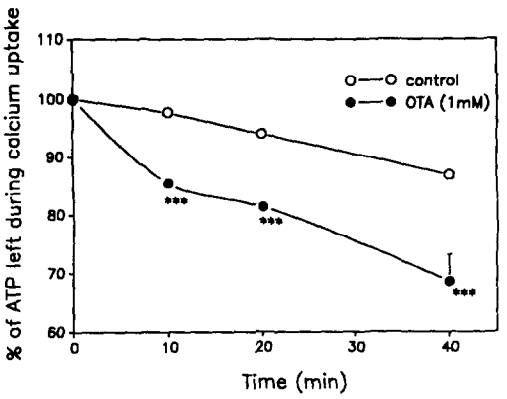

Fig. 9. Consumption of ATP during calcium uptake by microsomes preincubated with NADPH in the presence and absence of OTA. Microsomes were preincubated with NADPH ± OTA (1 mM), and the centrifuged microsomes were incubated with 5 mM ATP as described under calcium uptake (Fig. 1) except that $^{45}Ca^{2+}$ was omitted. At timed intervals 0.5-mL aliquots were withdrawn for determination of ATP levels by HPLC as described in Materials and Methods. Values are means ± SD from two experiments, one performed in quadruplicate and the other in duplicate. Key: (***) denotes a significant difference ($P < 0.001$) between control and OTA.

Table 1. Effect of OTA on phosphorylation of kidney microsomes

Treatment	[^{32}P] Bound (nmol/mg protein 10^6) Expt. 1	Expt. 2
In vivo		
Control	0.7	0.6
OTA (10 mg/kg, i.p., 2 hr)	1.7	1.5
Control	7.0	5.8
OTA (4 mg/kg, i.p. daily for 4 days)	2.9	2.5
In vitro		
Control (−NADPH)	1.2	1.4
+NADPH	0.3	0.2
OTA (1 mM)	0.6	0.7
OTA (1 mM) + NADPH	0.8	0.6

Microsomes were labeled with [γ-^{32}P]ATP and subjected to SDS–PAGE as described in Materials and Methods. Individual values from two separate experiments are shown.

Table 2. Effect of OTA administration on renal malondialdehyde levels

OTA treatment	MDA (nmol/mg protein)
Whole homogenate	
Control (solvent)	0.57 ± 0.14
5 mg/kg, i.p. (30 min)	0.30 ± 0.02*
5 mg/kg, i.p. (2 hr)	0.44 ± 0.04†
Cortex homogenate	
Control (solvent)	0.24 ± 0.06
0.5 mg/kg, i.p. (daily for 4 days)	0.26 ± 0.03
1 mg/kg, i.p. (daily for 4 days)	0.25 ± 0.01
2 mg/kg, i.p. (daily for 4 days)	0.29 ± 0.04
4 mg/kg, i.p. (daily for 4 days)	0.23 ± 0.04

MDA levels were measured either in whole homogenate or cortex homogenate as described in Materials and Methods. Values are means ± SD of duplicate determinations from 3 individual rats per treatment group. Results were analyzed by one-way analysis of variance and Bonferroni *t*-test as post-test.

* $P < 0.001$.

† $P < 0.05$.

in renal microsomal calcium pump activity appears to be the earliest enzymatic change reported in the kidney after OTA administration. De Witt *et al.* [43] observed a similar increase in renal endoplasmic reticulum calcium uptake activity after administration of cisplatin.

The inhibitory effect of NADPH on the renal cortex calcium pump activity and its partial reversal by OTA are interesting. Prasad *et al.* [44] previously observed a similar inhibitory effect of NADPH on rat liver microsomal calcium uptake activity and attributed it to an oxygenated cytochrome P450 complex which directly oxidized critical protein thiol groups since GSH was found to reverse the inhibitory effect of NADPH. These authors have suggested that activity of the microsomal calcium pump *in vivo* is maintained by the cytochrome P450 system which tends to oxidize sulfhydryls and thereby decrease activity while the enzyme thiol protein disulfide reductase reduces these protein disulfides to reactivate the pump [45]. On the assumption that a specific isozyme(s) of cytochrome P450 is responsible for the inhibition of the liver microsomal calcium pump and that a substrate/inhibitor for that particular isozyme would protect against inactivation, Srivastava *et al.* [46] concluded that few cytochrome P450 isozymes, most likely members of the CYPIIB and CYPIIIA family could be involved in the inhibitory effect. Possible involvement of the CYPIIB family in the reversal of the inhibitory effect of NADPH by OTA is consistent with the results of Hietanen *et al.* [47] who showed that phenobarbital pretreatment of rats induces OTA-4-hydroxylase activity.

The liver microsomal calcium pump activity has been shown to be very sensitive to oxidative damage and lipid peroxidation [20]. We have reported previously that OTA administration to rats increases ethane exhalation (an index of *in vivo* lipid peroxidation) [48] and inhibits the liver microsomal calcium pump activity [22]. Also, OTA addition to liver microsomes in the presence of NADPH greatly enhances lipid peroxidation and strongly depresses microsomal calcium uptake. Moreover, a good correlation was observed between inhibition of ATP-dependent calcium uptake and lipid peroxidation as judged by MDA formation [22]. An increase in the rate of calcium uptake by rat kidney cortex microsomes subsequent to OTA treatment suggests

that OTA probably does not induce lipid peroxidation in the kidney. In keeping with this, we were unable to demonstrate increased levels of lipid peroxides in the kidney subsequent to OTA dosing (Table 2). In fact, a sifnificant decrease in MDA levels was observed in kidneys of rats pretreated 30 min to 2 hr earlier with OTA, which is in agreement with the increase in the rate of calcium uptake observed.

The advent of oxidative stress *in vivo* often leads to increased levels of antioxidant enzymes which serve to protect the organism/tissue from the deleterious effects of reactive oxygen species and lipid peroxidation products [38]. Although we have shown [49] that in a reconstituted system consisting of NADPH, the flavoprotein NADPH-cytochrome P450 reductase and Fe^{3+}, OTA stimulates superoxide and hydroxyl radical formation, the lack of induction of any antioxidant enzyme together with the absence of increased MDA levels suggests that *in vivo* OTA may not produce significant quantities of these species in the renal cortex.

The inhibitory effect of OTA alone on calcium uptake *in vitro* could possibly be due to its ability to disrupt the integrity of the microsomal membrane. Ochratoxin A is known to bind tightly to proteins. Chu [50] showed that serum albumin binds two equivalents of OTA tightly. We have also observed that incubation of liver or kidney microsomes with [^{3}H]OTA results in the association of a significant amount of protein-bound radioactivity which could not be removed by repeated washing with trichloroacetic acid and organic solvents. Preliminary evidence using ESR spectroscopy also indicates that OTA is able to disturb the ordered structure of liver microsome vesicles containing spin-labeled fatty acids. However, it does not appear that *in vivo* OTA exerts a significant direct effect on renal cortex calcium uptake activity since an increase in this activity was observed after OTA administration.

It is interesting that *in vivo* OTA exerts opposite effects in liver and kidney in terms of microsomal calcium uptake. One possible explanation for this could be that OTA in the presence of cytochrome P450 might be exerting two effects that oppose each other. As a substrate for cytochrome P450, OTA would be expected to reduce the steady-state levels of the active cytochrome P450–oxygen complex and thereby decrease the inactivation of the pump. However, OTA can also stimulate lipid peroxidation both *in vivo* and *in vitro* by a process shown to be mediated by cytochrome P450 [51]. The onset of lipid peroxidation would be expected to inhibit the calcium pump activity. The calcium pump activity observed after OTA administration *in vivo* or addition *in vitro* may therefore be a reflection of the extent of these two processes. In the liver, lipid peroxidation may be dominant leading to an overall inhibition of the calcium pump activity [22]. The present data indicate that, under our experimental conditions, OTA does not appear to induce lipid peroxidation in the renal cortex. It is therefore possible that the increase in calcium uptake activity observed could be due to the inhibitory effect of OTA on the steady-state level of the active cytochrome P450–oxygen complex. However, the involvement of cytochrome P450 in OTA-dependent changes in microsomal calcium uptake remains to be demonstrated.

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