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PERTURBATION OF LIVER MICROSOMAL CALCIUM HOMEOSTASIS BY OCHRATOXIN A

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Abstract—The effect of ochratoxin A on hepatic microsomal calcium sequestration was studied both *in vivo* and *in vitro*. The rate of ATP-dependent calcium uptake was inhibited by 42–45% in ochratoxin A intoxicated rats as compared to controls. In the presence of NADPH, addition of ochratoxin A (2.5 to 100 μM) caused a concentration-dependent inhibition of calcium uptake (28–94%) by untreated rat liver microsomes. The rate of NADPH-dependent lipid peroxidation, measured as malondialdehyde formed, was also greatly enhanced by ochratoxin A. Various agents that inhibited ochratoxin A enhanced lipid peroxidation were also able to block the destruction of calcium uptake activity. Lipid peroxidation enhanced by ochratoxin A was also accompanied by leakage of calcium from calcium-loaded microsomes. These results suggest that ochratoxin A disrupts microsomal calcium homeostasis by an impairment of the endoplasmic reticulum membrane probably via enhanced lipid peroxidation.

Ochratoxin A (OA§), a mycotoxin consisting of a 5′-chlorinated 3,4-dihydro-3-methylisocoumarin moiety linked by an amide bond to L-β-phenylalanine, is produced by several species of *Aspergillus ochraceus* and *Penecillum*. The natural occurrence of OA in food and feed stuffs is widespread [1], and it is known to be highly toxic to animals [2–4]. The main pathological changes associated with OA toxicity are kidney and liver damage [5, 6]. Several authors have also reported the occurrence of renal and hepatic tumors in mice after ingestion of high doses of OA [7, 8].

Administration of OA to rodents alters a variety of biochemical parameters in liver, kidney and urinary tract [9–11]. OA is known to be metabolised by liver microsomes of rats [12] and rabbits [13]. Recently, we reported that administration of OA to rats or addition of OA to rat liver microsomes enhances lipid peroxidation *in vivo* and *in vitro* respectively [14]. Here, we report the perturbation of hepatic calcium homeostasis after OA administration or *in vitro* addition of OA to untreated rat liver microsomes. The results presented demonstrate that destruction of calcium pump activity in the liver endoplasmic reticulum may be an early manifestation of OA toxicity.

MATERIALS AND METHODS

Reagents. Ascorbic acid, ATP, BHT, catalase, DMSO, GSH, isocitric acid, isocitric dehydrogenase, metyrapone, α-naphthoflavone, $NADP^+$, OA, SOD, DL-α-tocopherol and TBA were purchased from the Sigma Chemical Co., St. Louis, MO, U.S.A. Desferal was obtained from Ciba-Geigy Canada Ltd., Dorval, P.Q. All other chemicals were of the highest grade commercially available.

Animal treatment. Male Sprague–Dawley rats (200 ± 10 g) were obtained from Canadian Hybrid Farms, Halifax, N.S., and maintained on standard laboratory rat chow and water *ad lib*. The rats were starved overnight before being used. OA (10 mg/kg body weight) was administered intraperitoneally (i.p.) in 20 mM sodium bicarbonate. Control rats received an equivalent amount of vehicle. Animals were killed 5, 10 and 30 min after treatment. Liver microsomes were prepared as described previously [15]. Protein concentration was determined by the method of Lowry *et al.* [16].

In vitro *incubations.* Two separate incubation procedures were employed to examine the *in vitro* effects of OA on untreated rat liver microsomes. For the first, liver microsomes (1.0 mg/ml) were incubated in 0.1 M phosphate buffer (pH 7.4) with a range of OA concentrations (0–100 μM in up to 5 μl of DMSO/2 ml incubation volume) in the presence of an NADPH regenerating system (consisting of 0.4 μmol $NADP^+$, 5 μmol $MgCl_2$, 5 μmol DL-isocitrate and 0.65 units of isocitric dehydrogenase per ml of incubation volume). Calcium uptake and lipid peroxidation were assayed at various time points as described below. For the second, microsomes (1.0–1.2 mg protein/ml) were preincubated with OA (0–100 μM) and an NADPH regenerating system in 0.1 M phosphate buffer for 40 min. At the end of the incubation, an aliquot from each incubation mix was assayed to determine the extent of lipid peroxidation. The remaining mixtures were centrifuged at 105,000 *g* for 30 min at 4° to recover the microsomes. Very little further lipid peroxidation occurred during recovery of the microsomes. Microsomal pellets were resuspended in imidazole–histidine buffer (30 mM

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§ Abbreviations: BHT, butylated hydroxytoluene; DMSO, dimethyl sulfoxide; GSH, reduced glutathione; MDA, malondialdehyde; OA, ochratoxin A; SOD, superoxide dismutase; TBA, 2-thiobarbituric acid; and TCA, trichloroacetic acid.

imidazole, 30 mM histidine, 100 mM KCl, pH 6.8) and assayed for calcium uptake as described below.

Measurement of calcium uptake. Calcium uptake activity in liver microsomes from control or OA-administered rats or in *in vitro* incubated microsomes (as described above) was measured by the method of Moore *et al.* [17] as described earlier [18]. Briefly, microsomes (0.15 mg protein/ml) were incubated at 37° with 30 mM imidazole, 30 mM histidine, 100 mM KCl, 5 mM $MgCl_2$, 5 mM ATP, 5 mM ammonium oxalate, 20 μM $CaCl_2$ and 0.1 μCi/ml $^{45}CaCl_2$. The medium was adjusted to pH 6.8 with imidazole and prewarmed to 37°, and the assay was initiated by the addition of microsomes. At various time intervals, 0.5-ml aliquots were filtered with the aid of a vacuum apparatus on prewetted glass microfibre filters (Whatman, type 934-AH; diameter, 24 mm; pore size, 0.2 μm). After rapid washing with 10 ml of cold buffer, the filters were placed in scintillation vials and air dried. Bound radioactivity was monitored in a Beckman LS-1801 liquid scintillation counter after addition of scintillation fluid (Beckman, Ready solv-HP) to the vials.

Measurement of lipid peroxidation. Lipid peroxidation was estimated by measuring the level of MDA formed using the TBA assay as described before [14]. Briefly, lipid peroxidation was terminated at various time intervals by transferring 0.5-ml aliquots of the incubation mixture into tubes containing 50 μl of 2% BHT in ethanol and 500 μl of 30% TCA. After addition of 500 μl of TBA (0.67%) and 500 μl of distilled water, the tubes were heated in a boiling-water bath for 15 min, cooled and centrifuged at 3000 rpm for 10 min. The absorbance of the MDA–TBA adduct was read at 535 nm against a blank corresponding to the incubation of microsomes alone. An extinction coefficient of 1.56×10^5 $M^{-1} cm^{-1}$ was used for the MDA–TBA adduct.

Enzyme assays. Microsomes exposed to OA-NADPH were also assayed for glucose-6-phosphatase, benzo[*a*]pyrene hydroxylase, and ethoxycoumarin-*O*-deethylase activities. Glucose-6-phosphatase activity was measured with glucose-6-phosphate (35 mM) in imidazole–histidine buffer for 30 min at 37°. The inorganic phosphate released was measured by the method of Fiske and Subbarow [19]. Benzo[*a*]pyrene hydroxylase and ethoxycoumarin-*O*-deethylase activities were measured as described by Nebert and Gelboin [20] and Shull *et al.* [21] respectively.

Statistical analysis. Statistical comparisons between groups were carried out using Student's *t*-test. A level of significance of $P < 0.05$ (two-tailed) was chosen.

RESULTS

Figure 1 shows the ATP-dependent calcium uptake activity measured at various incubation times in liver microsomes isolated from control rats and rats dosed 10 min earlier with OA. Calcium uptake was inhibited markedly (42–45%) in the OA-intoxicated animals. The inhibitory effect of OA on microsomal calcium uptake was noticed as early as 5 min after OA administration and lasted for at least 30 min after toxin administration (data not shown).

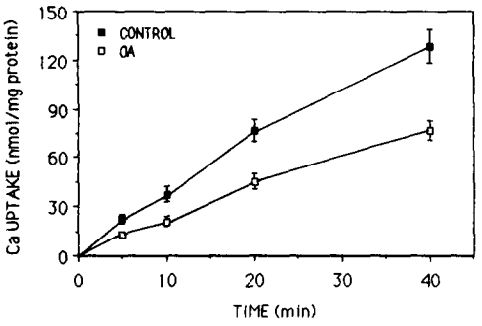

Fig. 1. $^{45}Ca^{2+}$ uptake by liver microsomes from control and OA-treated rats. Experimental details are given in Materials and Methods. Values are means ± SD from six rats. All OA values were significantly ($P < 0.05$) inhibited compared to control.

In vitro addition of OA to untreated rat liver microsomes also severely inhibited the calcium uptake activity (Fig. 2A). In the absence of OA, incubation of microsomes with an NADPH regenerating system for 40 min resulted in the uptake of 126.31 ± 12.82 nmol of Ca^{2+}/mg protein. Inclusion of 10 μM OA in the incubation medium resulted in an 80% decrease in calcium sequestration. In the absence of NADPH, the same concentration of OA (10 μM) did not produce a significant decrease in calcium uptake (Fig. 2A).

Inhibition of microsomal calcium uptake was associated with an enhancement of NADPH-induced lipid peroxidation (Fig. 2B). The increase in lipid peroxidation was directly dependent on the amount of OA added, indicating that the extent of lipid peroxidation could be controlled by the concentration of OA used.

In some experiments, lipid peroxidation (MDA formation) and calcium sequestration were measured simultaneously. In the presence of NADPH only (no OA), calcium uptake by microsomes increased linearly during the 40-min incubation period (Fig. 3A). When OA was included, the microsomes displayed a lower rate of calcium accumulation. However, this inhibitory trend in the presence of OA was much more significant after 20 min of incubation. These results suggest that inhibition of calcium sequestration may be related to, and dependent upon, the extent of lipid peroxidation. As seen in Fig. 3B, MDA production at the end of 20 min was between 1.29 and 3.16 nmol/mg protein in the presence of 5–100 μM OA. The corresponding values at the end of 40 min were 3.89–30.27 nmol MDA/mg protein.

Table 1 shows the effects of a variety of agents—active oxygen scavengers, antioxidants, GSH, an Fe^{3+} chelator and cytochrome P-450 inhibitors—on NADPH-dependent MDA formation and active calcium uptake by rat liver microsomes in the presence of 100 μM OA. In general, agents that inhibited or abolished the OA-stimulated lipid peroxidation also reduced or blocked the inhibitory effect on calcium sequestration. The lack of effect of catalase or SOD indicates that neither free superoxide anions

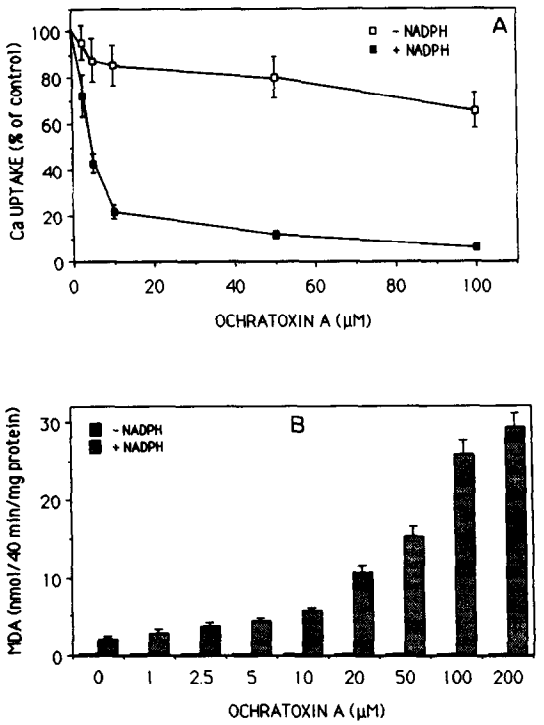

Fig. 2. Effect of OA on microsomal calcium uptake (A) and lipid peroxidation (B) in the presence or absence of NADPH. The initial incubations were carred out in 0.1 M phosphate buffer (pH 7.4) containing rat liver microsomes (1.0–1.2 mg protein/ml) and various concentrations of OA (0–200 μM) in the presence or absence of an NADPH regenerating system. The various concentrations of OA were added in DMSO (5 μl DMSO/2 ml incubation volume) which by itself neither enhanced lipid peroxidation nor inhibited the calcium uptake activity. After 40 min of incubation at 37°, an aliquot of the reaction mixture was analyzed for MDA levels. The rest of the incubation mixture was centrifuged, and the recovered microsomes, at a concentration of 0.15 mg protein/ml, were incubated for another 40 min at 37° for calcium uptake studies as described in Materials and Methods. Data in panel A are expressed relative to calcium uptake by unperoxidized microsomes (126.31 ± 12.82 nmol/mg protein) as 100. Each point is the mean ± SD of duplicate incubations from four independent experiments.

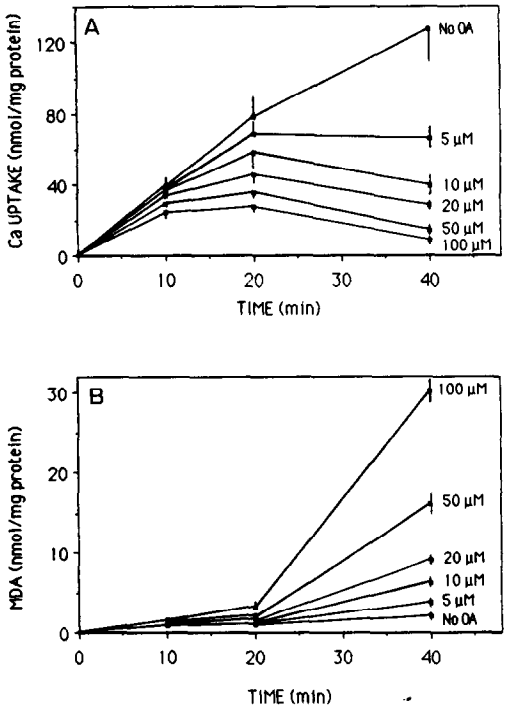

Fig. 3. Time course of calcium uptake (A) and MDA production (B) by liver microsomes in the presence of various concentrations of OA. Microsomes at a concentration of 0.25 mg/ml were incubated for 40 min at 37° with 100 μM $CaCl_2$, 5 mM ATP, 5 mM ammonium oxalate, 5 mM magnesium chloride, 5 mM sodium azide, $^{45}CaCl_2$ (0.6 μCi/ml), NADPH regenerating system, 100 mM potassium chloride, 30 mM imidazole, 30 mM histidine, (pH 6.8) and various amounts of OA. Other details are as described in Materials and Methods. Each point is the mean ± SD of duplicate incubations from three separate experiments.

nor hydrogen peroxide was involved. As expected, the antioxidant, BHT, and the iron chelator, desferal, virtually abolished lipid peroxidation and also the inhibitory effect on calcium sequestration. The cytochrome P-450 inhibitor metyrapone was without any effect, but α-naphthoflavone (100 μM) inhibited lipid peroxidation and restored calcium sequestration ability by about 85%.

Table 2 compares the effect of increasing OA concentrations on inhibition of calcium uptake and depression of microsomal enzyme activities such as benzo[*a*]pyrene hydroxylase, ethoxycoumarin-*O*-deethylase and glucose-6-phosphatase in peroxidizing microsomes in the presence of NADPH. At a peroxidation level of 5.74 nmol MDA/40 min/mg protein (10 μM OA), an 80% loss of calcium uptake activity was noticed, whereas loss of benzo[*a*]pyrene hydroxylase, ethoxycoumarin-*O*-deethylase and glucose-6-phosphatase activities was 27, 18 and 15% respectively. Thus, at low levels of MDA production, calcium uptake was the most severely affected of the indicators of microsomal enzyme activities examined.

DISCUSSION

Intracellular calcium compartmentation is governed by several transport systems operating in a highly regulated fashion. Loss of normal calcium homeostasis appears to be an early and critical event in the development of toxic cell injury [22]. An increase in cytosolic free calcium concentration resulting from the mobilization of intracellularly sequestered calcium and/or inhibition of cellular calcium extrusion seems to be critically linked to the development of cytotoxicity [23]. The endoplasmic reticulum plays an important regulatory role in maintaining intracellular calcium homeostatis and low cytosolic calcium levels [22–26]. The present report establishes that inhibition of the calcium pump of liver endosplasmic reticulum is an early event after administration of OA to rats. Within 10 min of OA

Table 1. Effects of various agents on ochratoxin A stimulated malondialdehyde formation and calcium uptake activity in rat liver microsomes

Additions to complete system	Lipid peroxidation (nmol MDA/mg protein/40 min)	Ca^{2+} uptake (nmol/mg protein/40 min)
(-OA)	1.98 ± 0.23	125.32 ± 8.57
None	28.82 ± 2.52	10.52 ± 0.88
BHT (25 μM)	1.56 ± 0.22	137.85 ± 9.52
Desferal (50 μM)	1.72 ± 0.31	125.88 ± 8.39
GSH (500 μM)	4.57 ± 0.38	92.95 ± 7.44
α-Naphthoflavone (100 μM)	4.01 ± 0.34	109.70 ± 9.26
Metyrapone (500 μM)	33.72 ± 2.94	12.59 ± 1.95
α-Tocopherol (250 μM)	20.75 ± 3.04	22.27 ± 2.03
Catalase (20 μg)	27.95 ± 2.91	9.62 ± 1.11
SOD (20 μg)	28.24 ± 3.11	9.55 ± 1.09

The complete system consisted of 1 mg microsomal protein/ml, 100 μM OA and an NADPH regenerating system in 0.1 M phosphate buffer (pH 7.4). The various agents were added as aqueous solutions or in 5 μl of methanol before addition of NADPH regenerating system which served to initiate lipid peroxidation. Incubations were carried out in duplicate at 37° for 40 min, and aliquots were taken for MDA measurement. The rest of the incubation mixtures were centrifuged, and the recovered microsomes were assayed for calcium uptake activity. Details are as described in Materials and Methods. Values are expressed as means ± SD from three separate experiments.

Table 2. Effect of OA addition on malondialdehyde formation, calcium uptake, benzo[*a*]pyrene hydroxylase, 7-ethoxycoumarin-*O*-deethylase and glucose-6-phosphatase activities in rat liver microsomes

OA concn (μM)	Lipid peroxidation (nmol MDA/mg protein/40 min)	Ca^{2+} uptake (% of control)	Enzymes activities (% of control)		
			Benzo[*a*]pyrene hydroxylase	Ethoxycoumarin-*O*-deethylase	Glucose-6-phosphatase
0	2.04 ± 0.37	100	100	100	100
2	3.45 ± 0.67	75.32 ± 9.12	103.15 ± 4.86	98.00 ± 8.56	99.32 ± 9.97
5	4.41 ± 0.35	43.34 ± 4.18	86.96 ± 9.66	95.00 ± 7.92	96.53 ± 9.97
10	5.74 ± 0.67	21.17 ± 3.12	73.33 ± 8.13	82.50 ± 7.65	85.53 ± 8.32
20	10.60 ± 1.04	18.83 ± 1.23	60.00 ± 5.19	75.32 ± 6.52	78.37 ± 5.39
50	15.44 ± 1.29	11.83 ± 1.75	16.52 ± 1.65	61.00 ± 5.32	65.33 ± 6.57
100	25.82 ± 1.93	6.11 ± 0.93	4.49 ± 0.35	32.50 ± 3.56	40.39 ± 4.57

The complete system consisted of 1 mg microsomal protein/ml, an NADPH regenerating system and various concentrations of OA in 0.05 M Tris–HCl buffer (pH 7.4). Incubations were carried out in duplicate at 37° for 40 min, and samples were taken for MDA measurement. The rest of the incubation mixtures were centrifuged, and the recovered microsomes were assayed for calcium uptake activity and other enzymes activities as described in Materials and Methods. Values are expressed relative to no OA addition as 100% and are means ± SD from three separate experiments. Actual values (in nmol/min/mg protein) are: Ca^{2+} uptake, 3.16 ± 0.32; benzo[*a*]pyrene hydroxylase, 115.58 ± 9.00; ethoxycoumarin-*O*-deethylase, 3.15 ± 0.28; and glucose-6-phosphatase, 240 ± 18.

administration, this activity was depressed by 42% (Fig. 1). Also, in *vitro* addition of OA to microsomes in the presence of an NADPH regenerating system caused a concentration-dependent inhibition of calcium uptake (Fig. 2A). Berndt *et al.* [27] reported that administration of the mycotoxins, OA and citrinin, to rats resulted in an increase in whole liver calcium content after 3 min. The inhibitory effect on microsomal calcium uptake activity and an early accumulation of calcium in liver have also been observed in carbon tetrachloride intoxicated rats [9, 28, 29]. These results suggest that a rise in calcium concentration and inhibition of endoplasmic reticulum calcium uptake may be common pathways in toxic cell death by various agents [23, 30].

The microsomal calcium sequestering system is very sensitive to oxidative damage. Several toxic agents, which can either directly or indirectly cause oxidant damage, can also decrease the activity of the calcium pump [31–38]. Studies by Orrenius and coworkers have suggested that the calcium pump activity is inhibited markedly by addition of peroxides to the incubation mixture [32–34, 36, 37]. Waller *et al.* [38] reported that microsomal calcium sequestration is depressed significantly by an enhancement of lipid peroxidation. In a previous study [14], we demonstrated that administration of a single dose of OA (5–10 mg/kg body weight) to rats enhances ethane exhalation (an index of *in vivo* lipid peroxidation) within 10 min and that this level steadily increases to about seven times that of control rats after 130 min. When OA was added to an incubation mixture consisting of liver microsomes from untreated rats and an NADPH regenerating system,

a dose-dependent increase in MDA formation was noticed (Fig. 2B). Thus, OA-enhanced lipid peroxidation may be a likely cause of the reduced ability of the endoplasmic reticulum to sequester calcium. This is consistent with observations that agents that inhibited or blocked OA-stimulated lipid peroxidation were also able to reduce or prevent the destruction of microsomal calcium pump activity (Table 1). Our results also demonstrate the sensitivity of microsomal calcium sequestration to OA-enhanced lipid peroxidation. At exceedingly low levels of lipid peroxidation, calcium uptake was inhibited more severely than either glucose-6-phosphatase or microsomal drug-metabolising enzymes (Table 2).

The disturbance of microsomal calcium accumulation during peroxidation enhanced by OA was also studied. The low level of peroxidation observed during the first 20 min of incubation was accompanied by only a small inhibition of calcium sequestration. However, extensive lipid peroxidation induced by OA during the next 20 min resulted in a severe decline in calcium accumulation (Fig. 3). This decline in accumulation could be the result of an alteration in the permeability of microsomes to calcium and/or to direct damaging effects on the pump itself. Calcium pump activity is known to be sensitive to changes in the phospholipid environment [39, 40]. Chien *et al.* [41] also reported decreased calcium sequestration in ischemic myocardial microsomes associated with altered phospholipid metabolism. In the case of OA intoxication, an alteration of membrane phospholipids may affect the calcium pump in a similar or direct manner. Other possibilities such as oxidation of a thiol group(s) which is necessary for Ca^{2+} ATPase activity in OA toxicity cannot be excluded (Table 1) [42].

In summary, our results suggest that OA causes a disturbance in endoplasmic reticulum calcium homeostasis which may be an early event in hepatotoxicity and which is correlated closely with minimal levels of OA-induced lipid peroxidation. This may perturb intracellular calcium homeostasis and possibly result in an alteration in calcium-stimulated enzyme activities and hormonal secretion which, in turn, can produce structural changes in the plasma membrane sufficient to allow an influx of extracellular calcium and cause cellular necrosis.

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