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Methoxychlor Inhibits Brain Mitochondrial Respiration and Increases Hydrogen Peroxide Production and CREB Phosphorylation

Rosemary A. Schuh,*,†,‡ Tibor Kristián,* Rupesh K. Gupta,†,‡ Jodi A. Flaws,†,‡ and Gary Fiskum*,‡,[1]

**Department of Anesthesiology, University of Maryland School of Medicine, Baltimore, Maryland 21201; †Department of Epidemiology and Preventive Medicine, University of Maryland School of Medicine, Baltimore, Maryland 21201; ‡Program in Toxicology, University of Maryland School of Medicine, Baltimore, Maryland 21201*

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The organochlorine insecticide methoxychlor (mxc) is an established reproductive toxicant that affects other systems including the central nervous system (CNS), possibly by mechanisms involving oxidative stress. This study tested the hypothesis that mxc inhibits brain mitochondrial respiration, resulting in increased production of reactive oxygen species (ROS). Oxygen electrode measurements of mitochondrial respiration and Amplex Red measurements of H_2O_2 production were performed with rat brain mitochondria exposed *in vitro* to mxc (0–10 μg/ml) and with brain mitochondria from mice chronically exposed *in vivo* to mxc (0–64 mg/kg/day) for 20 days by intraperitoneal injection. *In vitro* mxc exposure inhibited ADP-dependent respiration (state 3) using both complex I- and II-supported substrates. Similarly, state 3 respiration was inhibited following *in vivo* mxc exposure using complex I substrates. H_2O_2 production was stimulated after *in vitro* mxc treatment in the presence of complex I substrates, but not in mitochondria isolated from *in vivo* mxc-treated mice. Because previous studies demonstrated a relationship between oxidative stress and CREB phosphorylation, we also tested the hypothesis that mxc elevates phosphorylated CREB (pCREB) in mitochondria. Enzyme-linked immunosorbent assay (ELISA) measurements demonstrated that pCREB immunoreactivity was elevated by *in vitro* mxc exposure in the presence or absence of respiratory substrates, indicating that stimulation of H_2O_2 production is not necessary for this effect. These multiple effects of mxc on mitochondria may play an important role in its toxicity, particularly in the CNS.

Key Words: **methoxychlor; mitochondria; CREB; oxidative stress.**

INTRODUCTION

Organochlorines are a diverse group of synthetic chemicals including pesticides and industrial products that are persistent environmental pollutants due to their high lipophilicity and subsequent bioaccumulation in the food chain. The organochlorine pesticides dichlorodiphenoxytrichloroethane (DDT) and 1,1,1-trichloro-2,2-bis(*p*-methoxyphenyl)ethane (methoxychlor, mxc) have been shown in several studies to possess estrogenic properties resulting in adverse effects on the reproductive system in both animal models (Borgeest *et al.*, 2002; Cummings, 1997; Gray *et al.*, 1989) and cell lines (Chedrese and Feyles, 2001; Hodges *et al.*, 2000; Okubo *et al.*, 2004; Shekhar *et al.*, 1997). These studies indicate that exposure to organochlorine pesticides and related compounds are of concern in terms of human health.

Botella *et al.* (2004) determined the levels of several organochlorine pesticide residues in adipose tissue and blood samples from 200 women living in Southern Spain. The highest concentrations found were for 1,1-dichloro-2,2-bis(*p*-chlorophenyl)ethylene (*p,p′*-DDE), the major metabolite of DDT. Methoxychlor residues were also identified in this study, but at lower levels than the other pesticides tested. In addition, a recent study by Rudel *et al.*, (2003) determined the levels of 89 target chemicals including pesticides, designated as endocrine disruptors in urine samples, house dust, and indoor air from 120 homes in Cape Cod, MA. The results from the study indicate that DDT and mxc were present in at least 50% of the homes tested at relatively high concentrations.

In addition to their estrogenic properties, certain organochlorines including hexachlorocyclohexane (Sahoo and Chainy, 1998) and endosulfan (Kannan and Jain, 2003) have been demonstrated to play a role in cellular oxidative stress. Studies by Latchoumycandane and Mathur (2002) demonstrated depletion of antioxidant enzymes in mitochondria and microsomes from rat testis following exposure to mxc. Additionally, Chen *et al.* (1999) demonstrated increased superoxide production in rat liver mitochondria following exposure to the synthetic estrogen ethinyl estradiol. Other pesticides, including paraquat and rotenone, inhibit mitochondrial respiration and stimulate mitochondrial production of reactive oxygen species (ROS) in rat brain (Meyer *et al.*, 2004; Starkov *et al.*, 2004; Tawara *et al.*, 1996). The mitochondrial effects of environmental toxicants, *e.g.,* rotenone, are likely responsible for their

[1] To whom correspondence should be addressed at Department of Anesthesiology, University of Maryland School of Medicine, 685 W. Baltimore Street, MSTF 5–34, Baltimore, MD 21201. Fax: (410) 706-2550. E-mail: gfisk001@umaryland.edu.

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induction of neurodegeneration (Greenamyre *et al.*, 1999). Considering the epidemiological evidence that pesticide exposure may be linked to Parkinson's disease, a more extensive assessment of the effects of these toxicants on mitochondrial functions, including ROS production is needed.

Many studies have identified oxidative stress as an inducer of post-translational protein modification, resulting in transcriptional activation. For example, some studies indicate that oxidative stress during ischemia/reperfusion causes phosphorylation of Ca^{2+}/cAMP response element binding protein (pCREB) (Mabuchi *et al.*, 2001; Tanaka, 2001). In an earlier study, we determined that exposure of rat primary cortical and hippocampal neurons to the organophosphate insecticide chlorpyrifos increases pCREB immunoreactivity via a non-cholinesterase mechanism (Schuh *et al.*, 2002). CREB phosphorylation is implicated in the induction of transcriptional activity that stimulates the expression of antioxidant genes (Bedogni *et al.*, 2003). In addition to the effects of pCREB on nuclear gene transcription, mitochondrial gene expression may be under the control of mitochondrial-localized pCREB (Ryu *et al.*, 2003). pCREB is present in mitochondria from different tissues, including brain, and its phosphorylation state is regulated by Ca^{2+}, an important intracellular modulator of gene expression (Schuh *et al.*, 2005).

Although mxc and other endocrine disruptive compounds have been clearly identified as reproductive toxicants, other systems including the central nervous system (CNS) may also be targeted (Cooper *et al.*, 1999; Gore, A. C., 2002; Lafuente *et al.*, 2003). Furthermore, the mechanism(s) of action of these compounds within the CNS have not been fully investigated at the organelle level. Therefore, the primary objective of the present study was to determine the effects of *in vitro* and *in vivo* mxc exposure on brain mitochondrial respiration and ROS production. Considering the recent identification of mitochondrial CREB, we also tested the hypothesis that mxc increases mitochondrial CREB phosphorylation, possibly via stimulation of ROS production. The results of this study provide new insight into non-estrogenic effects of mxc that alter mitochondrial bioenergetics, producing oxidative stress. Mitochondrial pCREB is also identified as a new potential target of organochlorines.

MATERIALS AND METHODS

Chemicals and reagents. 1,1,1-trichloro-2,2-bis(*p*-methoxyphenyl)ethane (methoxychlor, mxc) was purchased from ChemService (West Chester, PA) in a powdered form and was 99% pure. All other reagents were purchased from Sigma (St. Louis, MO) unless otherwise stated.

Animals. Female CD-1 mice (25 g, 39 days old) were housed five animals per cage, and male Sprague-Dawley rats (300 g, 90 days old) were housed three animals per cage at the University of Maryland School of Medicine Central Animal Facility and provided food and water *ad libitum*. Animals were subjected to 12-h light:dark cycles. Mice were dosed via intraperitoneal injection with 16, 32, or 64 mg/kg/day mxc, or sesame oil (vehicle) for 20 continuous days. The mice were sacrificed when in estrus to minimize variability due to hormonal fluctuations within 24–72 h after the final mxc treatment. The *in vivo* mxc doses were selected based on earlier studies showing deleterious effects in the ovaries (Borgeest *et al.*, 2002, 2004). The University of Maryland School of Medicine Institutional Animal Use and Care Committee approved all procedures involving animal care, euthanasia, and tissue collection.

Mitochondrial isolation. Male Sprague-Dawley rat brains and female CD-1 mouse brains were rapidly dissected then further processed to isolate non-synaptosomal mitochondria using the Percoll isolation method described by Sims (1990). Briefly, after decapitation, the forebrain was rapidly removed and placed in ice-cold mannitol-sucrose (MS) buffer pH 7.4 (225 mM mannitol, 75 mM sucrose, 5 mM Hepes, 1 mg/ml fatty acid free BSA, 1 mM EGTA). The brain was homogenized then centrifuged twice at $1317 \times g$ for 3 min. After a further 10 min centrifugation at $21{,}074 \times g$, the pellet was resuspended in 15% Percoll (Amersham Biosciences, Piscataway, NJ) then layered on a discontinuous Percoll gradient and spun at $29{,}718 \times g$ for 8 min. The mitochondrial fraction was centrifuged at $16{,}599 \times g$ for 10 min then spun at $6668 \times g$ for 10 min. The mitochondrial pellet was resuspended in the above buffer but without BSA or EGTA. Protein concentrations were determined by the method described by Lowry *et al.* (1951).

Mitochondrial oxygen consumption. Oxidizable respiratory substrates consisting of either 5 mM L-malate plus 5 mM L-glutamate, 5 mM succinate plus 1 μM rotenone, or 0.02 mM N,N,N′,N′-tetramethyl-*p*-phenylenediamine (TMPD) plus 2 mM ascorbate and 1 μM antimycin A in potassium chloride buffer (30°C) containing 125 mM KCl ultrapure (Merck, Whitehouse Station, NJ), 20 mM Hepes, 2 mM K_2HPO_4, 0.01 mM EGTA, and 1 mM $MgCl_2$ (pH 7.0) were placed in a thermostatically controlled Clarke-type O_2 electrode (Hansatech Instruments, Norfolk, England). Isolated non-synaptosomal rat or mouse brain mitochondria (0.25 mg/ml) were added to the chamber and the rates of oxygen consumption were measured. For the *in vitro* mxc treatment studies, mxc (0–10 μg/ml) in dimethyl sulfoxide (DMSO) was added prior to mitochondrial addition. State 3 respiration was initiated 2 min after the addition of mitochondria by the addition of 0.8 mM ADP. Approximately 2 min later, state 3 respiration was terminated and state 4_o respiration (resting) was initiated with addition of 1.25 μg/ml oligomycin, an inhibitor of the mitochondrial ATP synthase. While the state 4_o respiration measured in the presence of oligomycin is not equivalent to the classical state 4 rate obtained after a small bolus of ADP is almost completely converted to ATP, the use of oligomycin eliminates the contribution of ATP cycling via hydrolysis by contaminating ATPases and resynthesis by the mitochondrial ATP synthase to state 4 respiration. The oligomycin-induced state 4_o rate of respiration is therefore a more specific indicator of the inner membrane proton leakiness. The maximal rate of uncoupled respiration was subsequently measured by titration with 54 nM carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazone (FCCP). The mitochondrial suspensions were centrifuged at $18{,}522 \times g$ for 3 min and the pellet resuspended in lysis buffer (pH 7.4) containing 0.5% NP40 (USB, Cleveland, OH), 1% Triton X-100, 150 mM NaCl, 10 mM Tris, and 1% protease inhibitor cocktail (Calbiochem, San Diego, CA). The aliquots were stored at −70°C.

Mitochondrial membrane potential. Mitochondrial membrane potential changes in isolated non-synaptosomal brain mitochondria (0.25 mg/ml) were followed qualitatively by monitoring the fluorescence of tetramethyl rhodamine methyl ester (TMRM, Molecular Probes, Eugene, OR), a cationic lipid-soluble probe that accumulates in energized mitochondria. Fluorescence intensity was measured in a Hitachi F-2500 fluorescence spectrophotometer (Tokyo, Japan) using the 549 nm wavelength for excitation and the emission wavelength set at 580 nm. An increase in fluorescence represents dequenching of TMRM when the probe is released into the medium upon mitochondrial depolarization. Briefly, the potassium chloride buffer (30°C) mentioned above was used, with addition of TMRM (100 nM) and oxidizable respiratory substrates consisting of 5 mM L-malate plus 5 mM L-glutamate. Following mitochondrial addition, sequential mxc (total amount 10 μg/ml) and FCCP (54 nM) were added.

Mitochondrial hydrogen peroxide production. Hydrogen peroxide (H_2O_2) production from isolated non-synaptosomal mitochondria from rat

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and mouse brains was measured fluorimetrically utilizing Amplex Red (Molecular Probes, Eugene, OR) as previously described (Starkov and Fiskum, 2003). Briefly, the potassium chloride buffer (30°C) mentioned above was used, with addition of 5 U/ml horseradish peroxidase, 40 U/ml Cu, Zn superoxide dismutase, and 1 μM Amplex Red. Measurements were initiated prior to addition of mxc and mitochondria to identify background rates. Methoxychlor (0–10 μg/ml) in DMSO was added to the cuvette prior to mitochondria in studies using rat brain mitochondria. After mitochondrial addition, the oxidizable substrates 5 mM L-malate plus 5 mM L-glutamate were added. Adenosine diphosphate (0.8 mM) was added a minute later, followed by oligomycin (1.25 μg/ml). When malate/glutamate were used as substrates, 1 μM rotenone was added, following oligomycin treatment. For experiments using alternative substrates, 1 μM rotenone was added followed by 5 mM succinate and 1 μM antimycin A addition after the oligomycin. Detection of H_2O_2 production was measured as an increase in fluorescence of Amplex Red dye at 585 nm with the excitation wavelength set at 550 nm. The dye response was calibrated with addition of a known amount of H_2O_2 (1 nmol). The concentration of the H_2O_2 stock was calculated from light absorbance at 240 nm employing $E_{240} = 43.6\ M^{-1}\ cm^{-1}$.

Experiments using DMSO (vehicle control) alone were performed for all the procedures mentioned above and DMSO was determined to have no effect on the parameters measured. The DMSO concentrations in all experiments were kept below 0.5% (data not shown).

pCREB immunoreactivity. Mitochondrial samples were assessed using an enzyme-linked immunosorbent assay (ELISA kit; BioSource International, Camarillo, CA) that recognized pCREB phosphorylated at serine 133. pCREB levels were determined according to the manufacturer's protocol.

Statistical analysis. Data are expressed as means ± SE, and the comparisons between experimental groups were made with SPSS statistical software (SPSS, Inc., Chicago, IL) using a regression analysis (test for trend). Statistical significance was assumed at $p < 0.05$.

RESULTS

Methoxychlor Alters Rat Brain Mitochondrial Respiration and Membrane Potential

After isolation of non-synaptosomal mitochondria from rat forebrain, we determined whether and how mxc affects respiration. In untreated mitochondria, ADP addition to the mitochondrial suspension in the presence of the oxidizable substrates malate and glutamate initiated state 3 respiration (Fig. 1A). Addition of the mitochondrial ATP synthase inhibitor oligomycin reduced the rate of O_2 consumption to that of state 4_o respiration, limited by the proton permeability of the inner membrane. In a few experiments, the rate of respiration was measured in the presence of the protonophore uncoupler FCCP, which stimulated respiration due to rapid futile cycling of protons across the inner membrane and collapse of the electrochemical gradient (Fig. 1A). The rate of respiration in the presence of FCCP is limited by the rate of electron transport rather than the ATP synthase or adenine nucleotide translocase activities, which can, under some circumstances, limit the rate of state 3 respiration. The lower trace in Figure 1A details control conditions without mxc addition, whereas the upper trace demonstrates that the presence of mxc (10 μg/ml) resulted in a 48% inhibition of state 3 respiration and a 43% increase in the state 4_o rate. Methoxychlor also inhibited

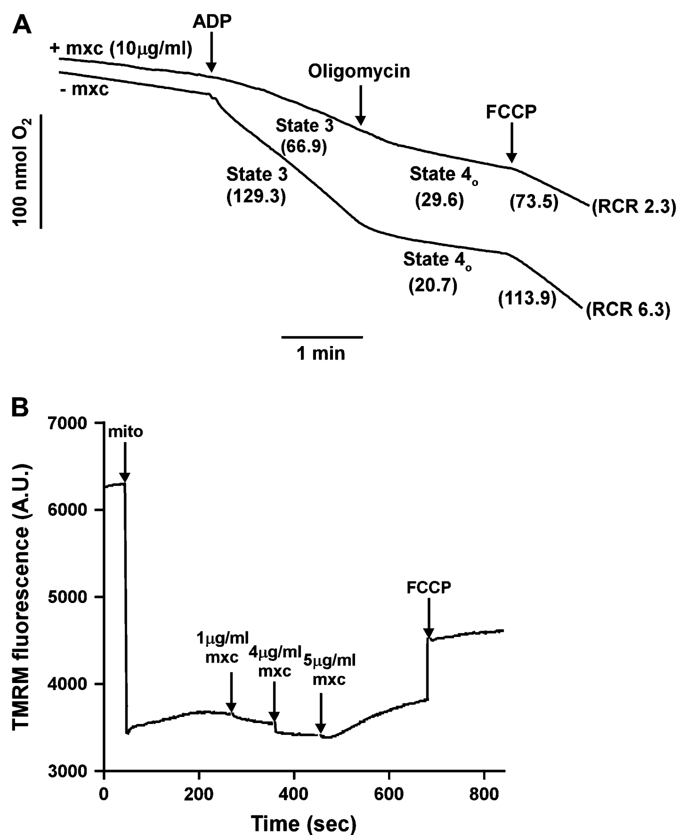

FIG. 1. Oxygen electrode measurements of respiration and fluorescence measurements of membrane potential using isolated rat brain mitochondria. A. Representative traces of mitochondrial oxygen consumption ± *in vitro* methoxychlor (mxc, 10 μg/ml) treatment in the presence of L-malate (5 mM), L-glutamate (5 mM), ADP (0.8 mM) to initiate state 3 respiration, and oligomycin (1.25 μg/ml) to induce state 4_o respiration. Maximal, uncoupled respiration was initiated with the protonophore carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazone (FCCP, 54 nM). The traces are representative of three to six separate experiments. B. Representative trace of changes in mitochondrial membrane potential ($\Delta\Psi$) following sequential additions of mxc (total amount 10 μg/ml) in the presence of L-malate (5 mM) plus L-glutamate (5 mM). Maximal $\Delta\Psi$ in uncoupled mitochondria was initiated with FCCP (54 nM). The trace is representative of three separate experiments. A.U., arbitrary units.

FCCP-stimulated respiration by 35%, suggesting a site of action at the electron transport chain rather than the ATP synthase.

The finding that 10 μg/ml mxc both inhibits state 3 respiration and stimulates state 4_o respiration suggests that mitochondrial membrane potential could be impaired. Qualitative, fluorescent TMRM measurements of mitochondrial membrane potential were performed under the same conditions used for the respiratory measurements, except that ADP was absent (Fig. 1B). Using suspensions of isolated mitochondria, depolarization causes an increase in TMRM fluorescence due to dequenching upon release of the fluorophore from mitochondria into the medium. After sequential additions of mxc totaling 10 μg/ml, the TMRM fluorescence increased toward

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the level obtained in the presence of FCCP; however, no mitochondrial depolarization was apparent at total doses of either 1 or 5 μg/ml.

The dose–response relationships for mxc and mitochondrial respiration are shown in Figure 2. The mxc doses used in these experiments were based on the studies of Miller *et al.* (2005) assessing the effects of mxc on apoptosis *in vitro*. Exposure to mxc (0–10 μg/ml) significantly reduced state 3 respiration when electron transport chain complex I substrates malate and glutamate were present ($p < 0.001$ compared to vehicle control) (Fig. 2A). In the presence of the complex II substrate succinate and the complex I inhibitor rotenone, mxc also demonstrated significant inhibition of state 3 respiration ($p < 0.001$) (Fig. 2A). Complex I-dependent respiration appeared more sensitive to inhibition than that of complex II (5 μg/ml mxc resulted in a 40% inhibition of respiration on malate plus glutamate compared to a 19% inhibition for succinate plus rotenone). Ascorbate plus TMPD were used to donate electrons to cytochrome c and then through complex IV to O_2 to probe for any effects of mxc on this distal portion of the electron transport chain. No significant effect on state 3 respiration following addition of mxc was observed under these conditions ($p = 0.61$, Fig. 2A).

After addition of oligomycin, *in vitro* mxc treatment produced a small but significant increase in state 4_o respiration in the presence of the complex II-linked substrate succinate ($p < 0.001$ as compared to vehicle control; Fig. 2B). No significant effects of mxc on state 4_o respiration were observed when using either malate plus glutamate or ascorbate/TMPD as oxidizable substrates ($p = 0.147$ and $p = 0.333$, respectively; Fig. 2B). Despite the lack of an effect on state 4_o respiration in the presence of malate plus glutamate, mxc caused a dose-dependent reduction in the respiratory control ratio (RCR) ($p < 0.001$ compared to vehicle control; Fig. 2C). For example, in the presence of mxc at 1 and 10 μg/ml, the RCR values were 3.38 ± 1.04 and 2.21 ± 0.54, respectively, compared to 6.41 ± 1.22 in the absence of mxc (Fig. 2C).

In Vitro *Methoxychlor Treatment Increases ROS Production by Rat Brain Mitochondria*

Because inhibition of mitochondrial respiration can, under some circumstances, result in increased production of ROS, we next determined whether mxc stimulated mitochondrial ROS production. As described previously (Starkov and Fiskum, 2003), fluorescent Amplex Red measurements of H_2O_2 were made with isolated brain mitochondria exposed to subsequent additions of malate plus glutamate, ADP, oligomycin, and rotenone (Fig. 3A and B). The presence of 10 μg/ml mxc resulted in an approximately sevenfold increase in H_2O_2 production during state 3 respiration (Fig. 3B) as compared with control (Fig. 3A).

Since mxc inhibition of respiration was greatest in the presence of complex I substrates, we hypothesized that the

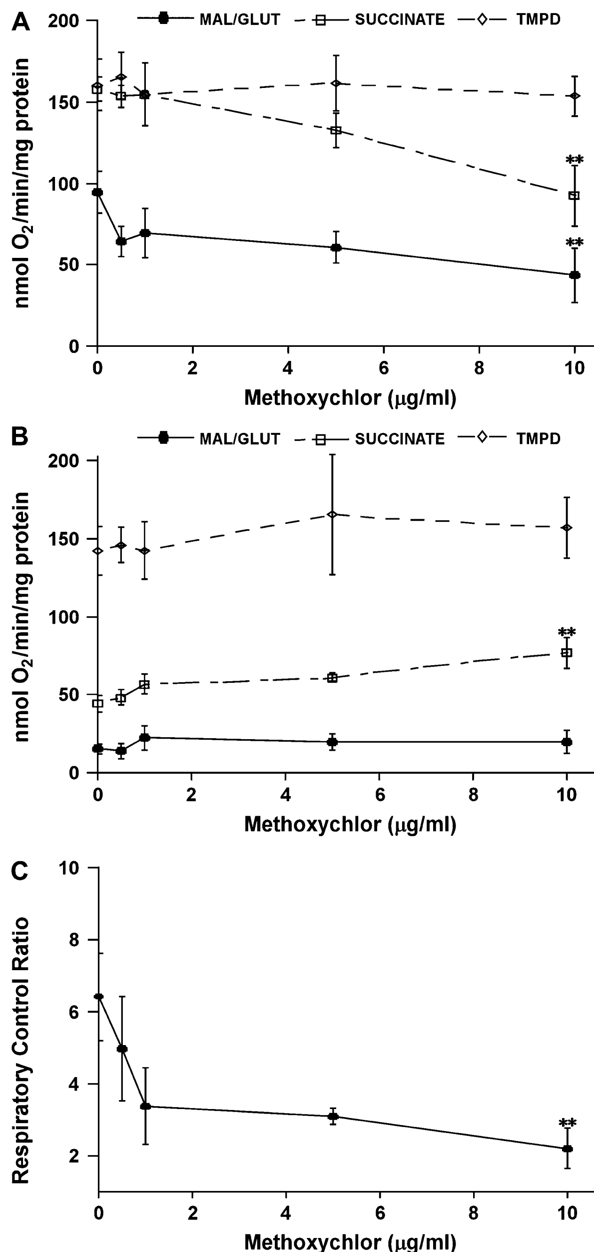

FIG. 2. Dose-dependent effects of *in vitro* methoxychlor treatment on rat brain mitochondria respiring on different oxidizable substrates. A. Mean state 3 oxygen consumption rates following exposure of isolated mitochondria to *in vitro* mxc (0–10 μg/ml) in the presence of either L-malate (5 mM) plus L-glutamate (5 mM); succinate (5 mM) and rotenone (1 μM); or N,N,N′,N′-Tetramethyl-*p*-phenylenediamine (TMPD, 0.02 mM), ascorbate (2 mM), and antimycin A (1 μM), measured as shown in Figure 1A. (B) Mean state 4_o oxygen consumption rates following addition of 1.25 μg/ml oligomycin were as shown in Figure 1A. Data are expressed as mean oxygen consumption rates (nmol oxygen/min/mg mitochondrial protein) and represent the mean ± SEM of three to six separate experiments. **Significantly different ($p < 0.001$) from control. C. Mean respiratory control ratios following exposure of isolated mitochondria to *in vitro* mxc (0–10 μg/ml) in the presence of L-malate (5 mM) plus L-glutamate (5 mM). Data are expressed as the ratio of state 3 rates:state 4_o rates and represent the mean ± SEM of three to six separate experiments. **Significantly different ($p < 0.001$) from control.

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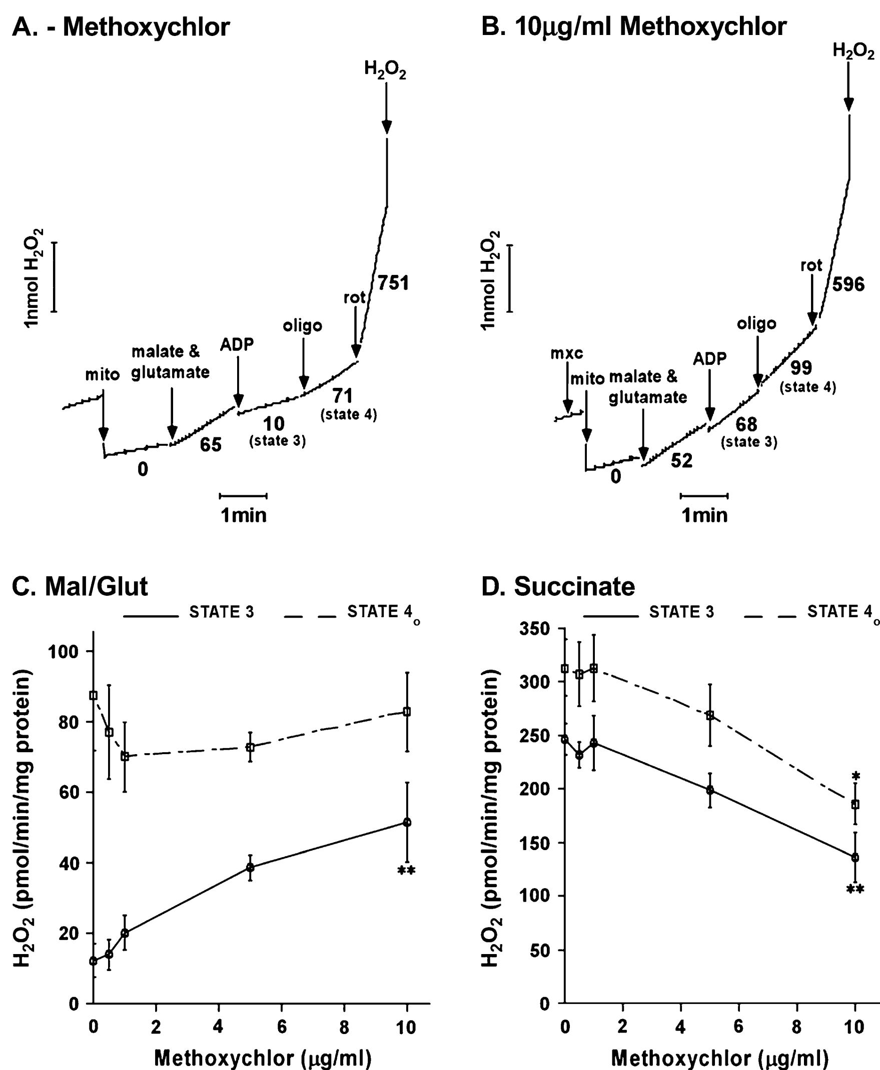

FIG. 3. Fluorescent Amplex Red measurements of H_2O_2 production in isolated rat brain mitochondria exposed *in vitro* to methoxychlor. Representative spectrofluorometer measurements of H_2O_2 production (A) without mxc or (B) plus mxc (10 µg/ml) in the presence of L-malate (5 mM) plus L-glutamate (5 mM), ADP (0.8 mM) to initiate state 3 respiration, and oligomycin (1.25 µg/ml) to induce state 4_o respiration. Mean H_2O_2 production rates after exposure of actively respiring mitochondria to mxc (0–10 µg/ml) in the presence of complex I-linked substrates (C) or complex II-linked substrates (D). Values represent mean H_2O_2 production rates (pmol/min/mg mitochondrial protein) ± SEM of three to four separate experiments. *Significantly different ($p < 0.01$); **significantly different ($p < 0.001$) from control.

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effect of mxc on ROS production would be more pronounced with malate plus glutamate than with succinate in the presence of rotenone. The rate of H_2O_2 production at state 3 respiration with malate plus glutamate as substrates increased significantly with increasing doses of mxc as compared with vehicle control ($p < 0.001$; Fig. 3C). In the presence of oligomycin (state 4_o), mxc had no significant effect on mitochondrial H_2O_2 generation ($p = 0.65$; Fig. 3C). In contrast to the stimulatory effect of mxc on ROS production with malate plus glutamate, mxc caused a significant reduction in succinate-supported ROS generation under both state 3 and state 4_o respiration ($p < 0.001$ and $p < 0.01$, respectively; Fig. 3D).

Effect of Chronic Methoxychlor Exposure in Mice on Brain Mitochondrial Respiration and H_2O_2 Production

Considering the effects of mxc on mitochondrial respiration and ROS production *in vitro*, experiments were performed to probe for possible effects of mxc on brain mitochondria *in vivo*. Female CD-1 mice were treated with mxc (0–64 mg/kg/day) in sesame oil via ip injection for 20 consecutive days prior to the mitochondrial isolation. This dose range was used because it produces follicular atresia (Borgeest *et al.*, 2002, 2004) and oxidative injury to the testes (Latchoumycandane and Mathur, 2002). State 3 respiration with the complex I-linked substrates malate and glutamate was significantly lower for brain mitochondria isolated from the mxc-treated mice compared

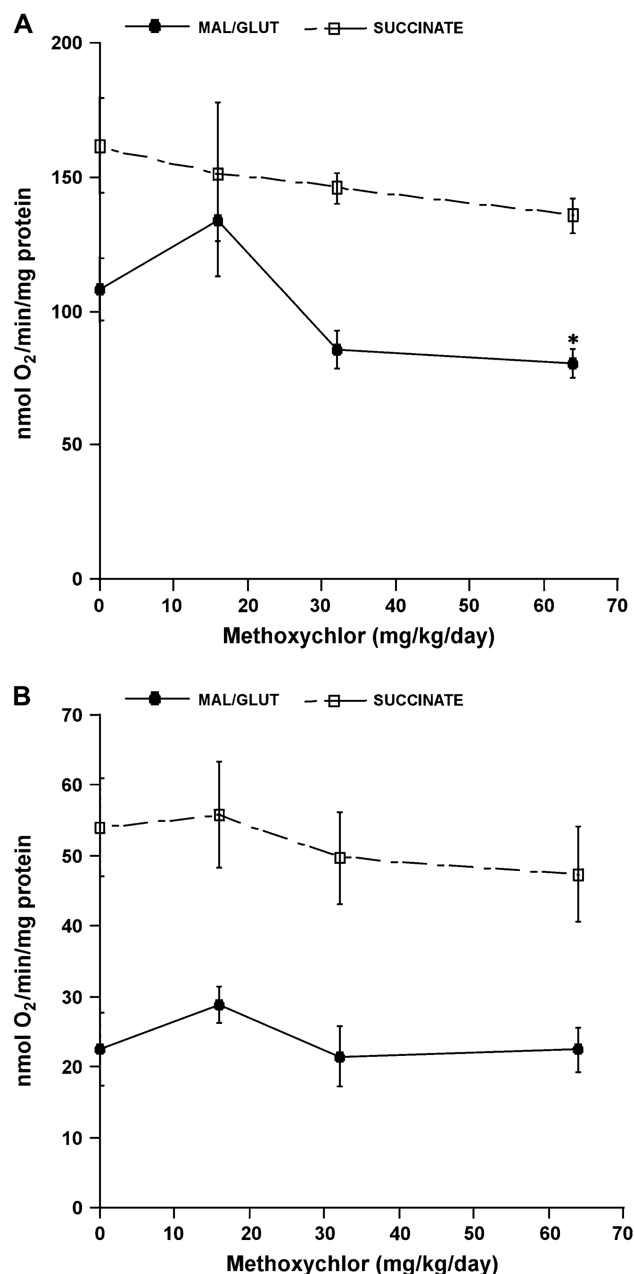

FIG. 4. Effects of *in vivo* methoxychlor treatment on mouse brain mitochondria respiring on different oxidizable substrates. A. Mean state 3 oxygen consumption rates in the presence of either L-malate (5 mM) plus L-glutamate (5 mM) or succinate (5 mM) and rotenone (1 μM) following *in vivo* mxc treatment (0–64 mg/kg/day) prior to mitochondrial isolation. B. Mean state 4_o oxygen consumption rates following addition of 1.25 μg/ml oligomycin. Data are expressed as mean oxygen consumption rates (nmol oxygen/min/mg mitochondrial protein) and represent the mean ± SEM of four separate experiments per treatment group. *Significantly different ($p < 0.05$) from control.

to those treated with the drug vehicle ($p < 0.05$; Fig. 4A). There was a similar trend toward inhibition for respiration using the complex II-linked substrate succinate with rotenone ($p = 0.08$; Fig. 4A). No significant changes in state 4_o respiration were observed with either complex I- or complex II-linked substrates ($p = 0.19$ and $p = 0.56$, respectively; Fig. 4B).

Because *in vivo* mxc treatment resulted in the inhibition of brain mitochondrial respiration, we then determined whether *in vivo* treatment also results in stimulation of H_2O_2 production by isolated mitochondria. No significant differences in H_2O_2 production were observed across treatment groups with either complex I-linked (Fig. 5A), or complex II-linked substrates (Fig. 5B).

In Vitro *Methoxychlor Treatment Increases the Phosphorylation State of Brain Mitochondrial Ca^{2+}/cAMP Response Element Binding Protein (CREB)*

Since it is well established in the literature that CREB can be phosphorylated and therefore activated following oxidative stress, we tested the hypothesis that CREB is a downstream target following mxc inhibition of mitochondrial respiration and stimulation of ROS production. At the end of the experiments measuring the effects of mxc on respiration, the mitochondrial suspensions were centrifuged and the mitochondrial pellet retrieved for ELISA measurements that are specific for the phosphorylated form of CREB (pCREB). As anticipated based on the stimulation of ROS production by mxc in the presence of malate plus glutamate, mxc also caused a significant increase in mitochondrial pCREB immunoreactivity ($p < 0.05$; Fig. 6A). Methoxychlor also caused a significant elevation of pCREB immunoreactivity in the presence of succinate plus rotenone, even though it did not stimulate ROS production under these conditions ($p < 0.001$; Fig. 6A).

To further assess the effect of mxc on CREB phosphorylation, mitochondria were incubated in the absence and presence of mxc in the absence of exogenous respiratory substrates, a condition that virtually eliminates mitochondrial ROS formation (Fig. 3A and B). Additionally, ATP (3 mM) was present to act as a phosphate source for CREB phosphorylation. In the presence of ATP and the absence of respiratory substrates, pCREB immunoreactivity was significantly elevated by mxc ($p < 0.001$, Fig. 6B), whereas mxc had no effect on pCREB in the absence of ATP. These results indicate that while mxc increases mitochondrial pCREB immunoreactivity, the stimulation of mitochondrial ROS production by mxc is not required to trigger this response.

Similar measurements were performed with the mitochondrial samples obtained from the *in vivo* mxc-treated mouse experiments. No significant effects of *in vivo* mxc treatment on pCREB immunoreactivity were present following incubation of mitochondria in the presence of complex I- or complex II-linked substrates ($p = 0.124$ and $p = 0.897$, respectively; Fig. 6C).

DISCUSSION

This study is, to our knowledge, the first to demonstrate the effects of methoxychlor on brain mitochondrial respiration and

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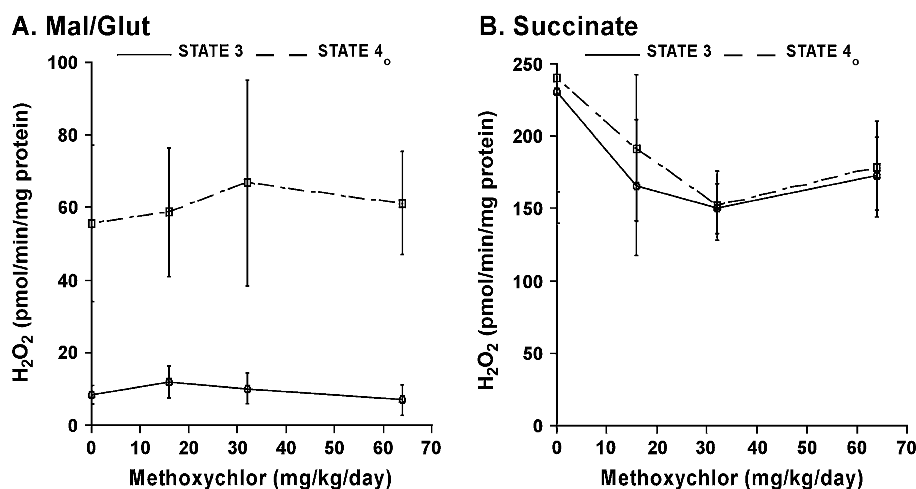

FIG. 5. Fluorescent Amplex Red measurements of H_2O_2 production in isolated mouse brain mitochondria treated *in vivo* with methoxychlor. Mean H_2O_2 production rates following *in vivo* mxc treatment (0–64 mg/kg/day) prior to mitochondrial isolation in the presence of actively respiring mitochondria utilizing complex I-linked substrates (A) or complex II-linked substrates (B). Values represent mean H_2O_2 production rates (pmol/min/mg mitochondrial protein) ± SEM for four separate experiments per treatment group. No significant differences were observed between treatment groups and vehicle controls.

production of ROS. Our results comparing the effects of mxc on mitochondrial O_2 consumption using oxidizable substrates that deliver electrons to different locations within the electron transport chain further define the regions of this pathway that are most affected by mxc. *In vitro*, mxc treatment significantly inhibits ADP-stimulated (state 3) respiration in the presence of the complex I-linked substrates malate plus glutamate and the complex II-linked substrate succinate (Fig. 2A), but it has no effect on O_2 consumption when measured in the presence of ascorbate plus TMPD, which deliver electrons to complex IV via cytochrome c (Fig. 2A). In addition, non-synaptosomal mitochondria isolated from mxc-treated mice exhibit significantly inhibited state 3 respiration in the presence of complex I-linked substrates but not in the presence of succinate plus rotenone (Fig. 4A). Thus, electron flow through complex I and, to a lesser extent complex II or III, is sensitive to inhibition by mxc treatment *in vitro* whereas only flow through complex I is affected by mxc treatment *in vivo*.

In addition to the respiratory inhibition most evident using complex I-dependent substrates, *in vitro* mxc treatment significantly increases state 4_o respiration in the presence of succinate plus rotenone (Fig. 2B). However, no significant effect on state 4_o respiration was observed across treatment groups in mitochondria isolated from *in vivo* mxc-treated mice (Fig. 4B). Thus, because state 4_o mitochondrial O_2 consumption in the presence of the ATP synthase inhibitor oligomycin is limited by the rate of H^+ influx across the inner membrane, stimulation of state 4_o respiration by mxc is likely due to a nonspecific increase in the ion permeability of the inner membrane caused by this lipophilic compound. This uncoupling effect is not as apparent in the presence of malate plus glutamate, because it is counteracted by more extensive respiratory inhibition. This interpretation is supported by the finding that mxc significantly lowers the respiratory control ratio measured in the presence of these substrates. The effects of mxc on respiratory coupling are also reflected by the partial loss of mitochondrial membrane potential observed at 10 µg/ml mxc (Fig. 1B). In summary, mxc is a respiratory inhibitor, particularly in the presence of complex I-linked substrates, and it is also a relatively mild respiratory uncoupler. The dual adverse actions of mxc on mitochondrial respiration indicate that it has the potential to induce cell death through metabolic failure or through adverse effects on respiration-linked activities, *e.g.*, superoxide formation.

Other toxicants, *e.g.*, DDT and paraquat, which inhibit the normal flow of electrons through the mitochondrial electron transport chain, also have the potential for increasing mitochondrial ROS production (Byczkowski and Tluczkiewicz, 1978; Tawara *et al.*, 1996). In our experiments, the rate of mitochondrial H_2O_2 production in the presence of malate plus glutamate and ADP was increased by more than 300% in the presence of 10 µg/ml mxc (Fig. 3C). The stimulation observed under state 3, but not state 4_o conditions is consistent with the state 3–specific respiratory inhibition observed with mxc. Maximal ROS production in the presence of complex I–dependent substrates is observed in the presence of a saturating concentration of the pesticide rotenone (Fig. 3A). The observation that mxc slightly inhibits the ROS production in the presence of rotenone (Fig. 3B) indicates that the sites of action of these toxicants within complex I are different and mxc acts at a redox site proximal to the site targeted by rotenone. Methoxychlor inhibits rather than stimulates H_2O_2 production when using the complex II substrate succinate (Fig. 3D). As mitochondrial ROS generation is regulated by the mitochondrial membrane potential through its influence of the redox state of mitochondrial electron transport chain components,

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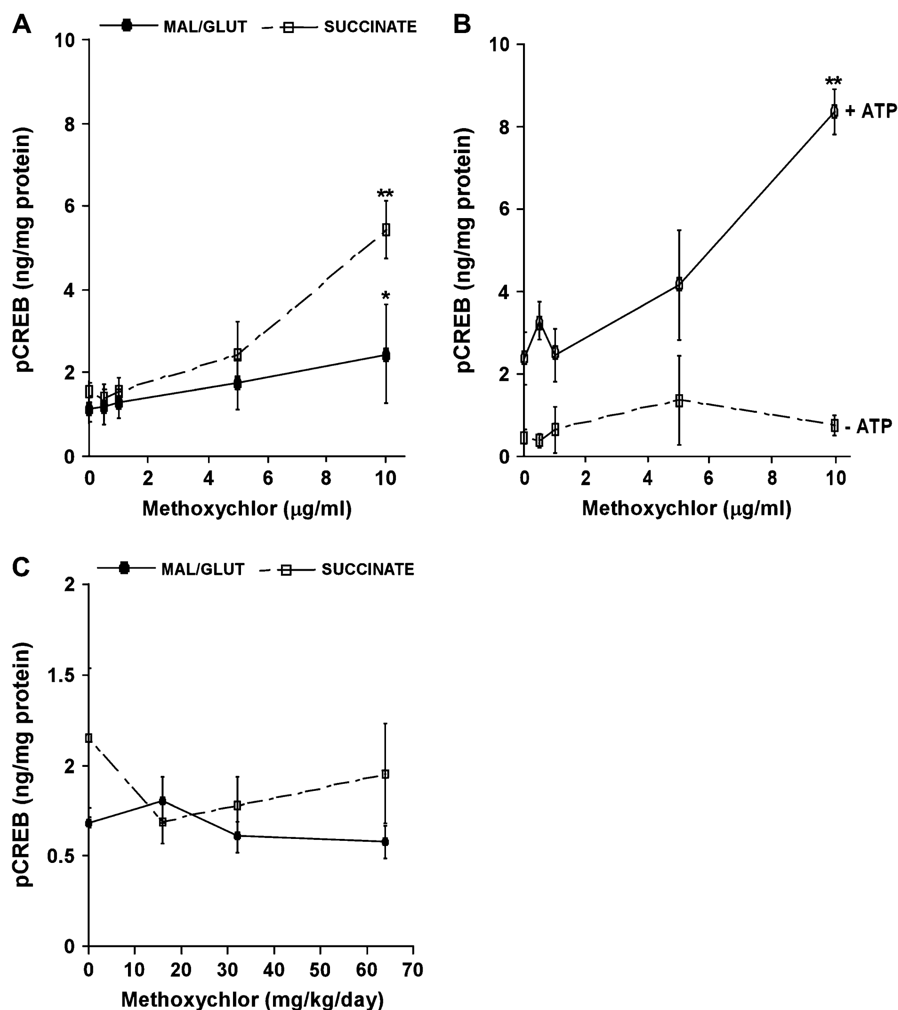

FIG. 6. Effects of methoxychlor on mitochondrial pCREB. A. pCREB levels (ng/mg mitochondrial protein) as assessed by ELISA following exposure of respiring rat brain mitochondria to *in vitro* mxc (0–10 µg/ml) treatment in experiments such as those shown in Figure 2A and B. Mitochondrial pCREB levels after incubation of rat brain mitochondria with mxc (0–10 µg/ml) in the absence of respiratory substrates and in the absence or presence of ATP (3 mM). C. pCREB levels (ng/mg mitochondrial protein) as assessed by ELISA after *in vivo* mxc (0–64 mg/kg/day) treatment in respiration experiments shown in Figure 4A. Values represent the means ± SEM for three to six separate experiments. *Significantly different ($p < 0.05$); **significantly different ($p < 0.001$) from control.

inhibition of succinate-supported H_2O_2 production is explained by the mild membrane depolarization known to inhibit ROS formation (Starkov and Fiskum, 2003). In summary, our direct measurements of the effect of mxc on H_2O_2 production by isolated brain mitochondria support the hypothesis that the toxicity of mxc is due in part to oxidative stress caused by its inhibition of electron flow through complex I of the electron transport chain. Moreover, our results extend the findings of Latchoumycandane and Mather (2002) by demonstrating that mxc-induced oxidative stress is not limited to the male reproductive system.

Although mxc stimulates mitochondrial ROS production *in vitro*, the rate of H_2O_2 production by mitochondria isolated from mice following *in vivo* mxc treatment was unchanged compared to vehicle-treated controls (Fig. 5). The presence of BSA in the mitochondrial isolation medium likely depletes the mitochondria of any residual mxc present *in situ* at the time the brain is removed. Thus, any mitochondrial alterations observed following isolation are the sequelae from the effects of mxc *in vivo*. Because complex I is sensitive to inhibition by ROS (Hillered and Ernster, 1983), stimulation of mitochondrial ROS formation by mxc present in the brains of chronically treated mice may result in oxidative modifications to complex I that are manifested as inhibition of state 3 respiration in the presence of malate plus glutamate. The absence of elevated H_2O_2 production by mitochondria isolated from mxc-treated animals indicates that the degree or nature of respiratory inhibition observed after mitochondrial isolation is insufficient to cause a detectable stimulation of ROS production. It is also possible that the metabolites of mxc generated *in vivo* have direct or indirect effects on brain mitochondria that are different from what we characterized for mxc *in vitro*. Further

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studies are therefore necessary to determine the mechanisms of action of mxc on brain mitochondria *in vivo*.

A unique finding of this study is that *in vitro* mxc treatment increases the immunoreactivity of phosphorylated CREB within mitochondria at mxc concentrations similar to those that both inhibit respiration and stimulate mitochondrial ROS production. Several labs have documented the presence of CREB and pCREB in mitochondria (Cammarota *et al*., 1999; Schuh *et al.*, 2005; Ryu *et al.*, 2003), and we recently demonstrated how the mitochondrial CREB phosphorylation state is regulated by both physiological and pathological levels of Ca^{2+} (Schuh *et al.*, 2005). As oxidative stress can stimulate nuclear gene expression via an increase in cellular CREB phosphorylation, we hypothesized that the mitochondrial oxidative stress caused by mxc increases mitochondrial pCREB levels *in vitro*. After measurements of respiration, pCREB present within isolated brain mitochondria was measured. pCREB levels were significantly elevated in the presence of *in vitro* mxc-treated mitochondria respiring on either malate and glutamate or succinate (Fig. 6A). Because mxc inhibits ROS production with succinate as electron donor, we measured the effects of mxc on pCREB levels in the absence of oxidizable substrates where mitochondrial H_2O_2 production is negligible (Starkov and Fiskum, 2003). Methoxychlor increases mitochondrial pCREB immunoreactivity in the absence of respiration and ROS production, and the degree to which pCREB is elevated is greater than that observed in the presence of oxidizable substrates (Fig. 6B).

Because the results do not support the hypothesis that mxc-induced oxidative stress is responsible for increased mitochondrial CREB phosphorylation *in vitro*, the effect of mxc is likely due either to inhibition of a phosphatase or to activation of a kinase. We did not observe an effect of *in vivo* mxc treatment on mitochondrial pCREB immunoreactivity; however, the conditions used during the mitochondrial isolation procedure are not sufficient to clamp CREB phosphorylation at the state in which it exists *in vivo*. As studies implicate the cellular CREB pathway in the response of tissues other than brain to mxc and other organochlorines (Chuang and Chuang, 1998; Zhang and Teng, 2002), future experiments will determine if the phosphorylation state of mitochondrial and non-mitochondrial CREB in the brain are affected by mxc at the *in vivo* doses we found influence brain mitochondrial respiration.

The range of mxc concentrations that produce direct effects on mitochondrial respiration, ROS production, and CREB phosphorylation state are within the range of those that could feasibly exist *in vivo* at doses that elicit oxidative stress and cell death. The doses used *in vivo* range from approximately 20 to 200 mg/kg/day (Gray *et al.*, 1989). If the tissue concentrations generated at these doses are only 1% of these levels, the total concentration is in the range of 0.2–2.0 μg/ml, *i.e.*, comparable to the range of 0.5–10.0 μg/ml used in the *in vitro* mitochondrial experiments. It is impossible at this juncture to relate these levels to those that exist in humans, as virtually no data on mxc levels in human tissue are available.

It is well established that compromised mitochondrial respiration plays a role in initiation of apoptotic cascades. Additionally, several studies have suggested a relationship between defective energy metabolism and neurodegenerative diseases including Alzheimer's disease (Mutisya *et al.*, 1994) and Parkinson's disease (Greenamyre *et al.*, (1999). Thus, the findings that mxc inhibits brain mitochondrial respiration, stimulates ROS production, and increases mitochondrial CREB phosphorylation warrant further investigation into the role that endocrine-disruptive compounds may play utilizing non-estrogenic mechanisms of action, including those present within mitochondria.

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