Irreversible Binding and Adrenocorticolytic Activity of the DDT Metabolite 3-Methylsulfonyl-DDE Examined in Tissue-Slice Culture

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The persistent adrenocorticolytic DDT metabolite 3-methylsulfonyl-DDE ($MeSO_2$-DDE) was originally identified in Baltic grey seals, a population suffering from adrenocortical hyperplasia. In mice, $MeSO_2$-DDE induces mitochondrial degeneration and cellular necrosis in the adrenal zona fasciculata. In this study, we used precision-cut tissue slice culture to examine local CYP11B1-catalyzed irreversible binding of $MeSO_2$-DDE in the murine adrenal cortex. We also examined effects on steroid hormone secretion, histology, and ultrastructure. As determined by microautoradiography, selective binding occurred in zona fasciculata of slices exposed to $MeSO_2$-[^{14}C]-DDE. Quantification of binding by phosphorautoradiography revealed a 3-fold reduction of binding in slices co-exposed to the CYP11B1 inhibitor metyrapone. As measured by HPLC, corticosterone and 11-deoxycorticosterone secretion to the medium increased linearly for at least 24 hr. Addition of the ACTH analog tetracosactide caused an 8-fold increase in corticosterone secretion. Addition of metyrapone reduced corticosterone secretion 4-fold. Exposure of slices to $MeSO_2$-DDE (50 µM) reduced the rate of corticosterone secretion by 90% after 24 hr of incubation. As determined by electron microscopy, vacuolated mitochondria were present in zona fasciculata of slices exposed to $MeSO_2$-DDE (50 µM) for 24 hr. Our findings show that all effects of $MeSO_2$-DDE previously reported *in vivo* could be reproduced in adrenal slice culture *ex vivo*. This test system allows analysis of zone-specific irreversible binding and effects on steroid hormone secretion and target cell ultrastructure. We propose adrenal slice culture as a simple *ex vivo* test system with which to examine the adrenocorticolytic activity of xenobiotics in human and wild animal tissue. Key words: adrenal cortex, DDT, endocrine disrupters, irreversible binding, 3-$MeSO_2$-DDE, tissue-slice culture, toxicity. *Environ Health Perspect* 109:105–110 (2001). [Online 10 January 2001]

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The chlorinated insecticide DDT is a persistent environmental pollutant that undergoes long-range atmospheric transport and is biomagnified in food chains. Although its biological degradation in the environment is slow, DDT is biotransformed to numerous lipophilic and persistent metabolites that are found in human tissues and in wild mammals, birds, and fish. These degradation products include the dechlorinated metabolites DDD and DDE and the sulfur-containing DDE metabolite 3-methylsulfonyl-DDE ($MeSO_2$-DDE). All metabolites have a capacity to interact with the endocrine system and have deleterious effects on humans and experimental and/or wild animals (*1*).

o,p´-DDD and *p,p´*-DDD are known to be tissue-selective toxicants following local metabolic activation and irreversible protein binding in the adrenal cortex in several species, including human (*2*), dog (*3*), and mink (*4*). By virtue of its tissue-selective toxicity, *o,p´*-DDD is currently used as an adrenocorticolytic drug to treat adrenocortical carcinoma and Cushing's disease (*5*).

$MeSO_2$-DDE, along with a number of polychlorinated biphenyl (PCB)-derived methyl sulfones ($MeSO_2$-PCBs), was originally identified in the blubber of Baltic grey seals (*6*). These persistent chemicals subsequently have been found in human breast milk, blood, and adipose tissue, as well as in adipose tissue of arctic polar bears (*7–9*). Several of these aryl methyl sulfones are characterized by their cell- and tissue-specific distribution patterns in the body. In the search for target cells of chlorinated aryl methyl sulfones it was found that ^{14}C-labeled $MeSO_2$-DDE produces a high and specific accumulation in the adrenal zona fasciculata in mice (*10*). Unlike the $MeSO_2$-PCBs (polychlorinated biphenyls), which are irreversibly associated with specific PCB-binding proteins in their target cells (e.g., in nonciliated bronchiolar cells), $MeSO_2$-DDE is irreversibly bound in the adrenal zona fasciculata cells. $MeSO_2$-DDE has subsequently been shown to be a highly potent toxicant that induces mitochondrial degeneration and cellular necrosis following a CYP11B1-catalyzed metabolic activation in the adrenal cortex in mice (*11,12*).

Introduced in the early 1980s (*13*), precision-cut tissue-slice culture has attracted interest as a test system with which to examine drug metabolism *in vitro* (*14–17*). Recently the adrenal medulla has been examined with this method (*18,19*). We have not found any reports using precision-cut adrenal slices for studies of the cortical function. Some authors have used quartered adrenals or hand-cut adrenal slices (*20–24*). Our objective has been to develop and apply a test system based on tissue-slice culture that allows examination of $MeSO_2$-DDE and other metabolism-activated adrenocorticolytic agents in human and wild animal tissues. We now report on the use of precision-cut adrenal slices to examine metabolism-activated irreversible $MeSO_2$-DDE–binding, ultrastructural changes, and effects on glucocorticosteroid secretion in mouse and rat tissue. The results show that adrenal tissue-slice culture is a promising *ex vivo* test system for future studies on adrenal toxicants in human and wild animals.

Materials and Methods

Chemicals. 3-Methylsulfonyl-2,2´-bis(4-chloro-[^{14}C]-phenyl)-1,1´-dichloroethene ($MeSO_2$-[^{14}C]DDE; 13.4 mCi/mmol), unlabeled $MeSO_2$-DDE, and 2-(2-chlorophenyl)-2-(4-chloro-[^{14}C]-phenyl)-1,1-dichlorethane (*o,p´*-[^{14}C]DDD; 11.2 mCi/mmol) were prepared as previously described (*25,26*). DL[4,5-^{3}H]leucine (40 Ci/mmol) was purchased from Amersham Life Science (Amersham, England). The radiochemical purity was > 99% for all compounds. Tetracosactide (Synacthen Depot, 1 mg/mL) was obtained from Ciba (V. Frölunda, Sweden). Bovine serum albumin (BSA), dimethylsulfoxide (DMSO), and agarose (Type VII, low melting temperature) were obtained from Sigma (St. Louis, MO, USA). 2-Hydroxyethyl-methacrylate Technovit

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7100 was obtained from Kulzer (Wehrheim, Germany) and TAAB 812 resin from TAAB (Aldermaston, Berks, England). Osmium tetroxide came from Analytical Standards AB (Mölnlycke, Sweden) and uranyl acetate and lead citrate from Merck (Darmstadt, Germany). All liquids and dyes were from Merck except chloroform, from Prolabo (Paris, France). Liquid film NTB2 was purchased from Kodak (Rochester, NY, USA). Aquasafe 300 Plus was obtained from Zinsser (Frankfurt, Germany).

Animals. Female C57Bl mice (18–20 g) and Sprague Dawley rats (100–160 g) were obtained from Charles River, Uppsala, Sweden. The animals were kept on a 12 hr light:12 hr dark regimen and given a standard pellet diet (Lactamin R36; Stockholm, Sweden) and tap water *ad libitum*.

Preparation and incubation of tissue slices. Animals were killed with carbon dioxide. The adrenals were rapidly excised and kept in ice-cold phosphate-buffered saline (PBS) until embedding in 3% agarose. Particular care was taken to arrange the adrenals with the craniocaudal axis perpendicular to the sectioning plane. Precision-cut slices (200 µm) were prepared in a Krumdieck tissue slicer (Alabama Research and Development, Munford, AL, USA) in ice-cold PBS (*13*).

Slices of equal size cut through the central part of the adrenal were placed on titanium screen holders (two to four slices per holder). The holders were transferred to standard six-well plates containing fully supplemented Dulbecco's modified Eagle's Medium (FDMEM, 2.5 mL) supplemented with fetal bovine serum (FBS, 2%), gentamycin (0.1%), L-glutamine (2 mM), penicillin (100 U/mL), streptomycin (100 µg/mL), and 2-mercaptoethanol (50 µM; National Veterinary Institute, Uppsala, Sweden). Slices were placed in culture within 1 hr of excision. The plates were kept rotating (1 rpm) on an inclined plane in an incubator (95% air/5% CO_2 at 38°C), thus allowing the slices to alternate between medium and incubator atmosphere. Incubation time ranged from 3 to 60 hr.

To inhibit CYP-dependent enzyme activity in the slices, some incubation wells were supplemented with the CYP11B1 (11β-hydroxylase) inhibitor metyrapone (50 µM) and preincubated for 30 min before renewing the medium. The synthetic ACTH analog tetracosactide was added (11 nM) to stimulate ACTH-regulated enzyme activity in the slices. The labeled and unlabeled test substances ($MeSO_2$-DDE and *o,p´*-DDD) were added to the fresh incubation medium, dissolved in DMSO (not exceeding 0.5% of total volume) and also with metyrapone when applicable.

Protein synthesis. Slices (four slices per well) were incubated in FDMEM (2.5 mL) supplemented with ^{3}H-leucine (0.4 µCi/mL) for up to 96 hr. Slices and medium (1 mL) were sampled every 12 hr. After sampling, slices were washed in PBS and homogenized in distilled water (1 mL). We determined protein content using a fluorescence-based protein assay (*27*). Samples of homogenate (50 µL) were added in duplicate to a 96-well plate. The wells were then supplemented with PBS (85 µL, pH 7.8) and fluorescamine (140 µL) dissolved in acetonitrile 0.3 mg/mL) and kept at room temperature for 15 min. Fluorescence was then measured in a Fluostar microplate reader (SLT Labinstruments GmbH, Grödig/Salzburg, Austria) excitation at 390 nm and emission at 460 nm. We calculated protein content using a standard curve prepared with BSA (0–250 µg/mL) (*27*).

Incorporation of ^{3}H-leucine into protein was measured in aliquots of the homogenate (0.5 mL), to which 2 M potassium hydroxide (KOH, 0.5 mL) was added. After mixing, 1.5 M acetic acid (1 mL) was added and the samples were centrifuged (300*g*, 20 min). The protein pellets were washed with 1.5 M acetic acid and centrifuged twice as above, before being dissolved in 0.5 M sodium hydroxide (0.5 mL). The solution was neutralized with 2 M hydrochloric acid (125 µL) and an aliquot (500 µL) was dissolved in scintillation liquid (Aquasafe 300 Plus; 5 mL). Radioactivity was measured in a Tri-Carb 1900CA (Packard, Downers Grove, IL, USA) liquid scintillator counter.

Hormone analysis. Corticosterone, 11-deoxycorticosterone, and aldosterone concentrations in the medium were measured with HPLC using UV detection (241 nm). Medium (1 mL) was removed and steroid hormones were extracted twice with chloroform:methanol (2:1, 1.5 mL). The combined chloroform phases were evaporated to dryness, redissolved in acetonitrile (50%), and injected into the HPLC system (Lichrosorb RP 18 column, 20 cm, 5 µm particle size; Merck). The steroid products were separated using a linear gradient of 40–80% acetonitrile (1 mL/min) and mixed with 40% methanol over 25 min. The amounts of steroids were expressed as nmol/slice. The detection level of steroid hormones was 5 pmol/mL medium.

Autoradiography. **Microautoradiography**. Slices were incubated with $MeSO_2$-[^{14}C]DDE (7.5 µM, 0.1 µCi/mL) or *o,p´*-[^{14}C]DDD (6.3 µM, 0.1 µCi/mL) for 24 hr, as above. The labeled substances were added to the medium dissolved in DMSO to localize enzyme-catalyzed irreversible metabolite binding.

Following incubation, the slices were fixed overnight in buffered formaldehyde (4%). The fixed slices were dehydrated in an ethanol series (70%, 95%, and 100%) and embedded in methacrylate. Particular care was taken to orient the slices with the sliced plane parallel to the sectioning plane. In a two-step embedding procedure, the sections were fixed in the correct position with a minimal volume of methacrylate and then mounted on a plastic holder with the remaining methacrylate. The mounted slices were sectioned (2 µm) in a rotating microtome (HM 360; Mikrom Laborgeräte GmbH, Walldorf, Germany). Slides carrying the sectioned slices were dipped in NTB2 liquid emulsion (Kodak) diluted with an equal volume of distilled water. To enable localization of irreversible binding in metyrapone-treated mouse slices and rat slices, an exposure time of 60 weeks (4°C) was required to show clearly the localization. General exposure time was 6–10 weeks. Autoradiograms were developed, stained with toluidine blue, and examined in a Leica (DM RXE) light microscope. Photographs were taken with a digital camera (Leica, Wetzlar, Germany) and processed in Adobe Photoshop 5.5 (Adobe, San Jose, CA, USA). Selected autoradiograms were mounted and printed on a Fujix Pictrography 3000 (Fuji, Japan).

Phosphorautoradiography. Semi-quantification of tissue-bound radioactivity was performed by apposing tissue sections to imaging plates (BAS-IP MP 2040S; Fuji, Japan) for 14 days, before subjecting them to microautoradiography. The radioactivity in the labeled areas of the adrenal sections was recorded by reading the imaging plate in a phosphoimager (BAS 1500; Fuji, Japan) (*28,29*). For semiquantification of the tissue-bound radioactivity, we used a Macintosh-based bioimaging analyzer program (MacBAS, ver. 2.2; Fujifilm, Fuji, Japan).

To correlate radioactivity and metabolically active regions in the incubated slices, we marked the labeled areas of the images selectively at 1 pixel resolution (1 pixel = 100 µm). Values obtained were expressed as phosphostimulated luminiscence (PSL) minus background (BG) per square millimeter of 2-µm thick tissue sections [(PSL-BG)/mm^2].

Histopathology and electron microscopy. We added $MeSO_2$-DDE (dissolved in DMSO) to the wells in amounts corresponding to a final concentration in the medium of 50 µM. Following incubation, slices were embedded in methacrylate and prepared for light microscopy, as above. For reference purposes, some adrenal slices were fixed directly after sectioning. For transmission electron microscopy, slices were fixed overnight in glutaraldehyde (2.5% in 0.067 M N-cacodylate buffer). They were then treated with osmium tetroxide (1%, 2 hr at 4°C) rinsed in PBS buffer, dehydrated in ethanol as described above, treated with acetone, and embedded in TAAB 812 resin. After we selected areas to be examined, we cut ultrathin sections (50 nm)

with a diamond knife. Sections were placed on copper grids and counterstained with uranyl acetate (4%, 30 min) and lead citrate (0.1 M, 5 min), and then examined with a Philips EM 420 transmission electron microscope at 60 keV (Philips, Eindhaven, the Netherlands).

Statistical evaluation of data. All statistical analyses of hormone concentrations and bound radioactivity were made with a one-way analysis of variance (ANOVA) (using Dunnet's posttest) or linear regression test with GraphPad Prism software version 3.0 for Windows (GraphPad Software, San Diego, CA, USA).

Results

Protein synthesis. Adrenal slices exposed to tetracosactide maintained a constant rate of ^{3}H-leucine incorporation into protein [counts per minute (CPM) per milligram protein], which remained linear for 60 hr (Figure 1A).

Steroid hormone secretion. The rate of corticosterone secretion from nontreated adrenal slices remained constant for 30 hr. We could detect no secretion of 11-deoxycorticosterone from these slices.

In slices exposed to tetracosactide, accumulation of corticosterone and 11-deoxycorticosterone in the medium increased linearly for about 48 hr. Incubation for more than 48 hr reduced the rate of secretion of corticosterone and 11-deoxycorticosterone (Figure 1B). Tetracosactide exposure for 24 hr increased corticosterone content in the medium 8-fold ($p < 0.05$), compared with that in nontreated slices (Figure 2). Aldosterone content increased almost 4-fold ($p < 0.01$) under the same conditions (Figure 2).

In slices exposed to the CYP11B1 inhibitor metyrapone, the rate of corticosterone secretion was reduced about 4-fold during 30 hr of incubation, compared with nontreated slices. Exposure of slices to both metyrapone and tetracosactide for 24 hr produced no significant decrease in corticosterone secretion, compared with slices exposed to tetracosactide only. 11-Deoxycorticosterone secretion from slices exposed to both tetracosactide and metyrapone showed a 6-fold increase ($p < 0.01$), while aldosterone secretion was more than halved ($p < 0.01$), compared with slices exposed to tetracosactide only (Figure 2).

Slices exposed to both $MeSO_2$-DDE (50 µM) and tetracosactide showed a reduction in the rate of corticosterone secretion (90%) at time points exceeding 6 hr of incubation, compared with slices exposed to tetracosactide where the rate of corticosterone secretion remained constant for 30 hr of incubation (Figure 3). The levels of 11-deoxycorticosterone after 30 hr of incubation were similar for both treatments.

Autoradiography. As determined by light microscopy, autoradiograms of adrenal slices co-exposed to $MeSO_2$-[^{14}C]DDE and tetracosactide were characterized by a marked labeling of zona fasciculata (Figure 4 A,B). Labeling of zona glomerulosa, zona reticularis, and the adrenal medulla did not exceed that of the background. The localization of $MeSO_2$-[^{14}C]DDE in nontreated slices did not differ from that in tetracosactide-exposed slices. The images of tissue-bound radioactivity semiquantified with phosphorautoradiography matched the images of the microautoradiograms closely. Phosphorautoradiography showed no significant increase in amount of tissue-bound $MeSO_2$-[^{14}C]DDE in slices exposed to tetracosactide, compared with non-exposed slices. The $MeSO_2$-[^{14}C]DDE-derived labeling in zona fasciculata in slices exposed to both tetracosactide and metyrapone was reduced about 3-fold ($p < 0.01$), compared with slices exposed to tetracosactide only (Figure 4 C,D). Localization of CYP11B1 mRNA using *in situ* hybridization produced autoradiograms that corresponded well with that of $MeSO_2$-[^{14}C]DDE labeling (unpublished data). Corticosterone secretion from slices exposed to $MeSO_2$-(^{14}C)DDE (<7.5 µM) was roughly identical to that from nontreated slices after 24 hr of incubation.

Rat adrenal slices exposed to $MeSO_2$-[^{14}C]DDE for 24 hr showed very weak labeling of zona fasciculata after 60 weeks of exposure (Figure 4 E,F). The levels of radioactivity in these slices were too low to be semiquantified by phosphorautoradiography using 14 days exposure.

In contrast, microautoradiograms of mouse adrenal slices exposed to *o,p´*-[^{14}C]DDD showed a very weak but selective labeling of zona fasciculata and zona reticularis (data not shown). Labeling of zona glomerulosa and the adrenal medulla did not exceed the background level The radioactivity in these sections remained too weak to be semiquantified by phosphorautoradiography using 14 days exposure.

Light microscopy. Except for a slight swelling of zona fasciculata cells, no obvious

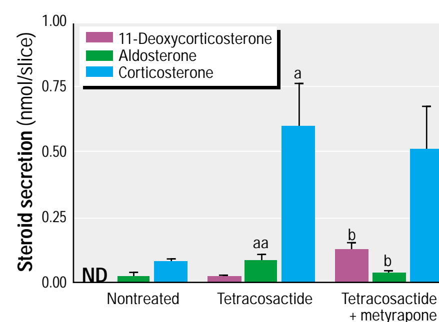

Figure 2. Steroid secretion from adrenal slices cultured for 24 hr. ND, not detectable. The secretion of 11-deoxycorticosterone, aldosterone, and corticosterone to the medium was increased by tetracosactide (11 nM) treatment (a, $p < 0.05$; aa, $p < 0.01$, compared with nontreated slices). The secretion of 11-deoxycorticosterone was increased and aldosterone was decreased by combined metyrapone (50 µM) and tetracosactide (11 nM) treatment (b, $p < 0.01$, compared with tetracosactide-treated slices). ($n = 5$, ± SE).

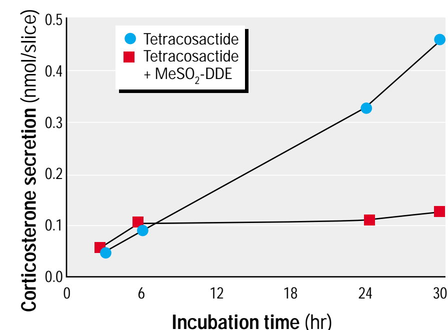

Figure 3. Effect of $MeSO_2$-DDE (50 µM) on corticosterone secretion. All time points represent secretion by four slices obtained from the same four animals. Corticosterone secretion to the medium was similar in all slices during the first 6 hr of culture. After 24 hr the $MeSO_2$-DDE (50 µM)-exposed slices showed a marked reduction in corticosterone secretion. Medium (1 mL) was removed from the wells after 3, 6, 24, and 30 hr and replaced with fresh medium; values were adjusted for dilution.

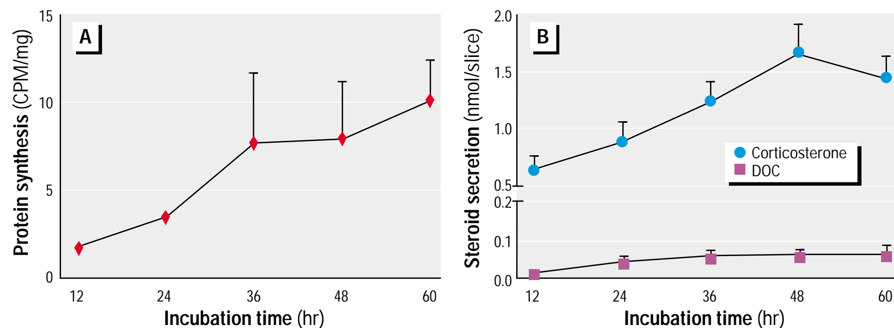

Figure 1. Protein synthesis and steroid hormone secretion determined in cultured adrenal slices incubated up to 60 hr in 5% CO_2 at 38°C. Medium and slices were sampled every 12 hr ($n = 5$); connecting lines with standard error of the mean are shown. (*A*) Protein synthesis was determined by constant exposure of the slices to ^{3}H-leucine (0.4 µCi/mL) in the medium. (*B*) Secretion of corticosterone and 11-deoxycorticosterone was measured in 1 mL of medium.

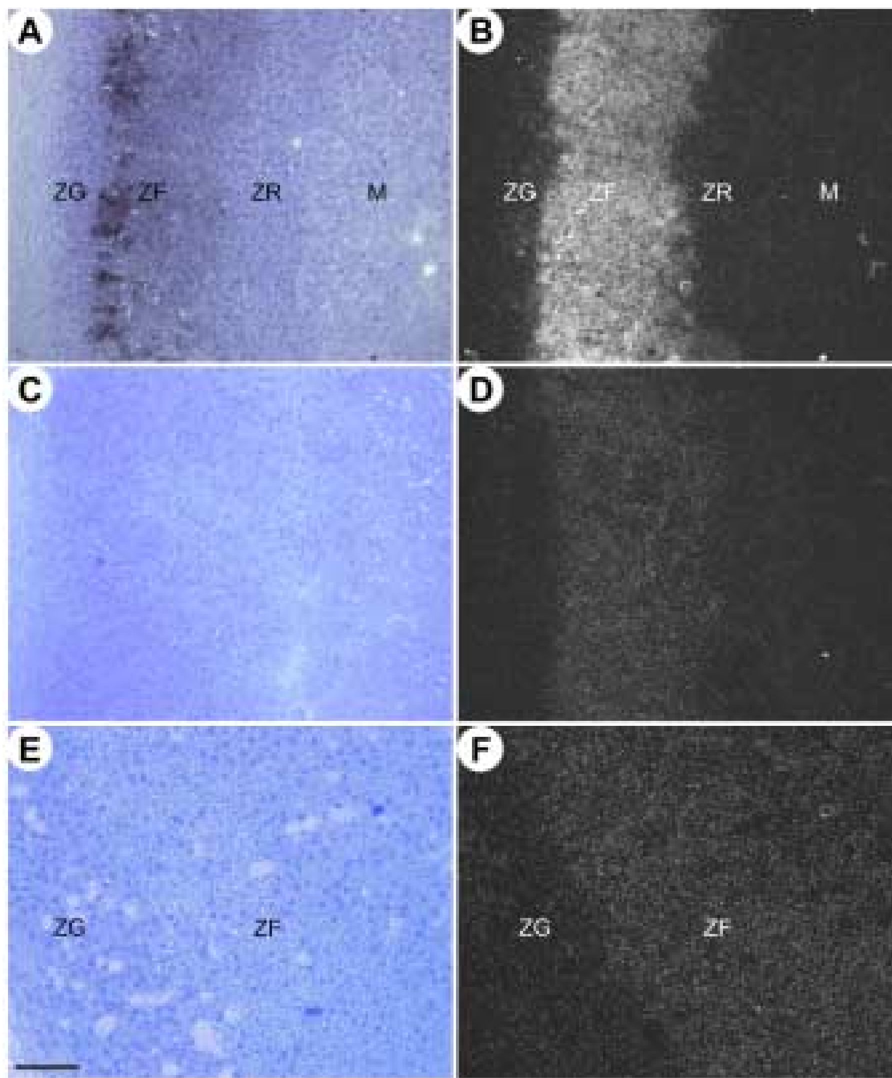

Figure 4. Specific tissue binding in $MeSO_2$-[^{14}C]DDE-exposed adrenal mouse (*A–D*) and rat (*E–F*) slices cultured in medium supplemented with tetracosatide (11 nM). Abbreviations: M, adrenal medulla; ZF, zona fasciculata; ZG, Zona glomerulosa; ZR, zona reticularis. $MeSO_2$-[^{14}C]DDE binding was confined to zona fasciculata in mouse adrenal slices, demonstrated by bright-field (*A*) and corresponding dark-field (*B*) images. $MeSO_2$-[^{14}C]DDE binding in mouse zona fasciculata was reduced after addition of the CYP11B1 inhibitor metyrapone (50 µM) (*C,D*). In rat adrenal slices $MeSO_2$-[^{14}C]DDE binding was very weak but distinct in zona fasciculata after exposure to tetracosactide (11nM) (*E,F*). Magnification x220; bar = 100 µm.

histologic changes could be observed after 24 hr of culture of tetracosactide-exposed slices, compared with noncultured slices fixed for histology immediately after slicing (Figure 5 A-B). In slices exposed to MeSO2-DDE, the nuclei in zona fasciculata cells appeared more condensed (Figure 5C).

Electron microscopy. Ultrastructural examination of slices fixed immediately after sectioning revealed numerous mitochondria and smooth endoplasmatic reticulum (SER) in zona glomerulosa and zona fasciculata cells. In slices kept 24 hr in culture, we observed a reduced amount of SER in both zones. Notably, the mitochondrial membranes were largely intact in zona glomerulosa and zona fasciculata. Slices incubated with MeSO2-DDE (50 µM) for 24 hr showed mitochondrial vacuolation in zona fasciculata. Mitochondria in zona glomerulosa remained largely intact and similar to those in nonexposed slices incubated for 24 hr (Figure 6 A–F).

Discussion

In the present study, we developed an *ex vivo* test system based on precision-cut adrenal slice culture to examine metabolism-dependent binding, ultrastructural changes, and effects on steroid synthesis in mouse adrenal tissue exposed to $MeSO_2$-DDE. The results showed that most metabolic and toxic events previously reported *in vivo* or *in vitro* can be reproduced *ex vivo.* In addition, the rate of steroid hormone synthesis in tetracosactide-exposed and nonexposed slices was readily recorded. The results suggest that adrenal tissue slice culture will become a useful test system for examination of metabolism-activated adrenal toxicants in both human and wild animal tissue.

The viability of cultured slices was defined largely by the toxicity-related test variables examined. As determined by a series of control experiments, nonexposed slices maintained functional activity for at least 48 hr. The lack of apparent histologic change in

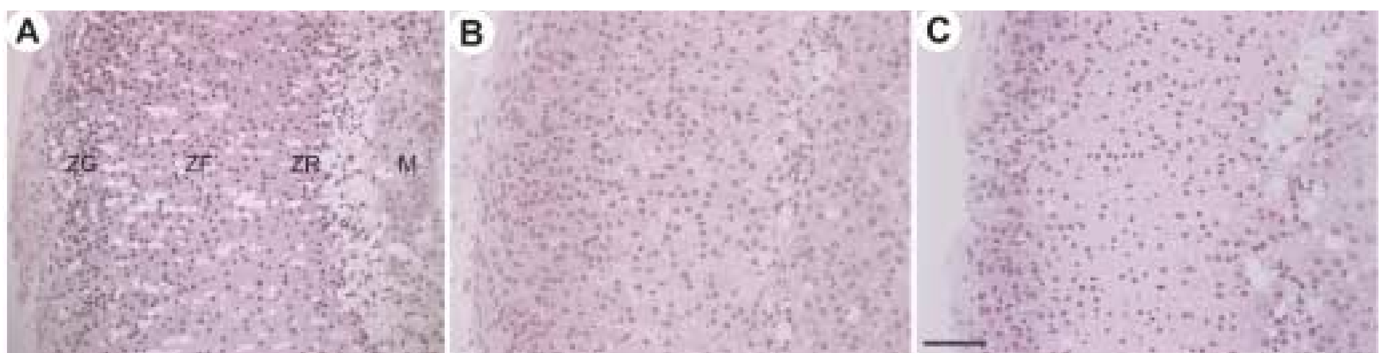

Figure 5. Histologic examination of a control section from an adrenal slice not placed in culture (*A*), a slice exposed to tetracosactide (11nM) (*B*), and a slice coexposed to $MeSO_2$-DDE (50µM) plus tetracosactide (11 nM) for 24 hr (*C*). Abbreviations: M, adrenal medulla; ZF, zona fasciculata; ZG, Zona glomerulosa; ZR, zona reticularis. A slight swelling of zona fasciculata cells could be observed after 24 hr, as compared with noncultured slices. In $MeSO_2$-DDE-exposed slices, the cell nuclei in zona fasciculata appeared more condensed. Hematoxylin-eosin–stained sections; magnification x220; bar = 100 µm.

the nonexposed slices following 24 hr in culture also supports the good viability of slices. Ultrastructural examination, however, revealed reduced numbers of SER vesicles after 24 hr in culture, showing that the metabolic functions of the SER could be affected at this time point. It is noteworthy that the mitochondrial membrane structure was largely intact, compared with that of tissue fixed immediately after slicing. This observation is particularly important because $MeSO_2$-DDE is activated by CYP11B1, an enzyme residing in the mitochondrial inner membrane of zona fasciculata cells.

Phosphorautoradiography proved a sensitive and quick tool to quantify levels of irreversible binding in the adrenal cortex. Combined with the exact localization of binding obtained by microautoradiography, phosphorautoradiography is an efficient technique with which to measure irreversibly bound adduct levels in restricted target cell populations in the cultured slices. The digitized autoradiograms representing sites of irreversible $MeSO_2$-[^{14}C]DDE binding correlated well with the localization of irreversibly bound radioactivity in conventional microautoradiograms. The irreversible $MeSO_2$-[^{14}C]DDE binding was confined to zona fasciculata, while zona glomerulosa, zona reticularis, and the adrenal medulla were devoid of bound radioactivity (above the background levels). These results are consistent with findings reported previously in mice dosed with $MeSO_2$-[^{14}C]DDE *in vivo*. They also confirm that CYP11B1 enzyme activity was maintained in the cultured slices.

The CYP11A1-catalyzed cholesterol side-chain cleavage is the first and rate-limiting step in corticosterone synthesis. CYP11B1 catalyzes the last step in this pathway but also, as mentioned, the metabolic activation of $MeSO_2$-DDE in the adrenal zona fasciculata (*12,30,31*). Since tetracosactide stimulates both CYP11A1 and CYP11B1 activity (*22,24,32–35*), this peptide would be expected to increase both corticosterone synthesis and irreversible $MeSO_2$-[^{14}C]DDE binding in the slice culture. The observed 8-fold induction of corticosterone secretion by tetracosactide was therefore expected and supports the conclusion that the cultured slices retained their functional stability throughout the experiment. Unexpectedly, however, no increased $MeSO_2$-[^{14}C]DDE binding in zona fasciculata was recorded by phosphorautoradiography. A possible explanation for this discrepancy could be that the increased concentration of 11-deoxycorticosterone induced by tetracosactide produced increased competition with $MeSO_2$-[^{14}C]DDE for the induced activating enzyme (*11*).

The reduced corticosterone secretion in $MeSO_2$-DDE–exposed slices may result from mitochondrial toxicity, but also from inhibition of CYP11B1 enzyme activity, as previously reported in adrenal Y1 cells (*31,36*). As demonstrated by electron microscopy, the mitochondrial membranes of zona fasciculata cells (the site of CYP11B1 localization) were vacuolated in the $MeSO_2$-DDE treated slices. The mitochondria in zona glomerulosa remained largely intact. This finding observed after 24 hr in culture and the delayed inhibition of corticosterone secretion suggests that toxicity was responsible (Figures 3 and 6). The mitochondrial changes observed were similar to those previously described *in vivo*, but the inhibition of corticosterone synthesis was more pronounced in the cultured slices. This difference may be explained by the pituitary feedback loop, which in the intact animal compensates for decreasing glucocorticoid serum levels by increasing the release of ACTH.

In vivo, the potent CYP11B1 inhibitor metyrapone blocks both synthesis of corticosterone from 11-deoxycorticosterone (*37*), and irreversible binding of $MeSO_2$-[^{14}C]DDE to adrenal homogenate and the mitochondrial fraction (*11*). Metyrapone treatment of slices would consequently be expected to inhibit both 11-deoxycorticosterone hydroxylation to corticosterone and metabolism of $MeSO_2$-[^{14}C]DDE to a reactive intermediate that becomes irreversibly bound in the zona fasciculata cells. In the present study, metyrapone did indeed reduce corticosterone synthesis about 4-fold and reduce irreversible $MeSO_2$-[^{14}C]DDE binding in zona fasciculata to a similar degree. A comparable inhibition of irreversible $MeSO_2$-[^{14}C]DDE binding was observed following

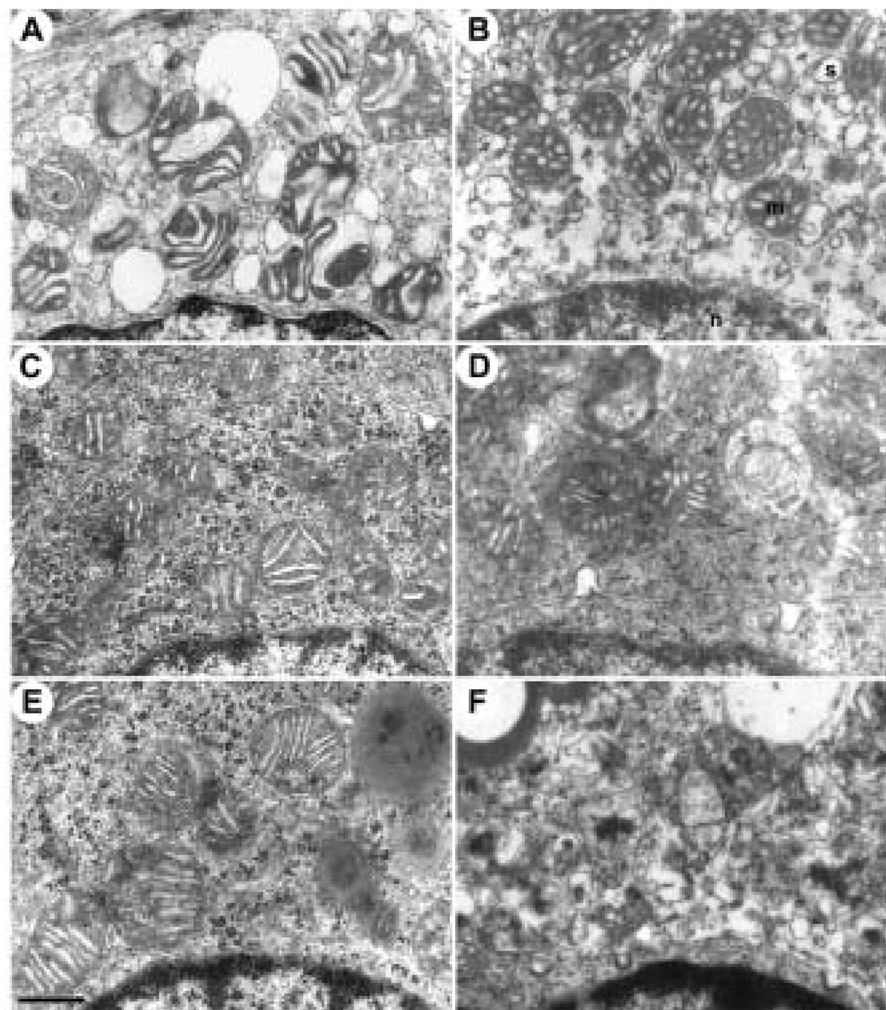

Figure 6. Ultrastructural examination of sections from adrenal slices. Abbreviations: m, mitochondria; n, nucleus; s, smooth endoplasmatic reticulum. Numerous mitochondria and SER characterize cells in zona glomerulosa (*A*) and zona fasciculata (*B*) in reference slices fixed immediately after sectioning. In slices kept 24 hr in culture (*C,D*), a reduced amount of SER is observed. Notably, the mitochondrial membranes are largely intact in zona glomerulosa (*C*) and zona fasciculata (*D*). Slices exposed to $MeSO_2$-DDE (50 µM) for 24 hr show mitochondrial vacuolation in zona fasciculata (*F*). Mitochondria in zona glomerulosa (*E*) remain largely intact and similar to those in slices incubated without $MeSO_2$-DDE for 24 hr (*C*). Magnification x27,000; bar = 0.5 µm.

exposure of slices to both tetracosactide and metyrapone. It is noteworthy, however, that the rate of corticosterone synthesis was not significantly reduced by metyrapone in tetracosactide-exposed cultures, in contrast to cultures where slices were exposed only to metyrapone. Notably, the concentration of the CYP11B1 substrate 11-deoxycorticosterone increased 6-fold in the co-exposed cultures, supporting an inhibition of CYP11B1. The failure to inhibit corticosterone synthesis by metyrapone may consequently be due to the increased substrate concentration for the enzyme. The remaining catalytic activity of CYP11B1 may suffice to maintain unchanged synthesis of corticosterone despite partial (competitive) inhibition of the enzyme by metyrapone. Moreover, metyrapone treatment halved the secretion of aldosterone.

The application of tissue-slice culture proved very useful for examining bioactivation and toxicity of $MeSO_2$-DDE in the mouse adrenal zona fasciculata *ex vivo.* To evaluate further the reliability of this *ex vivo* test system for studying metabolism-activated adrenal toxicants, we performed experiments with tissue from species known to be insensitive to $MeSO_2$-DDE and *o,p´*-DDD. Microautoradiograms showed a very weak binding of $MeSO_2$-[^{14}C]DDE to the rat zona fasciculata. Similarly, we observed very weak, but selective binding of *o,p´*-[^{14}C]DDD in zona fasciculata–reticularis in mouse adrenal slices. These findings are consistent with the low binding and toxicity of $MeSO_2$-DDE in rat and *o,p´*-DDD in mouse adrenal cortex *in vivo* (*38*). The finding that *o,p´*-DDD, unlike $MeSO_2$-DDE, was irreversibly bound in zona reticularis suggests that different CYP enzymes are involved in the metabolic activation of these adrenal toxicants.

The Baltic grey seal population suffered from adrenocortical hyperplasia and a suite of other pathologic lesions (*39,40*). $MeSO_2$-DDE was found in blubber from these animals. The adrenocorticolytic activity of $MeSO_2$-DDE has been characterized in subcellular adrenal fractions, cultured adrenal cells, and intact experimental animals. As shown by these studies, there are major differences in toxic potency between species, mice being highly sensitive whereas rats appear insensitive (*4,11,36,38,41*). Preliminary experiments with precision-cut human adrenal slices treated with $MeSO_2$-[^{14}C]-DDE and *o,p´*-[^{14}C]-DDD show consistent and promising results. Considering that $MeSO_2$-DDE is present in human milk and serum in high concentrations (*8,9*), a risk evaluation of possible human toxicity is called for.

In conclusion, we have shown that the bioactivation and mitochondrial toxicity of $MeSO_2$-DDE observed *in vivo* could be reproduced *ex vivo.* We have also demonstrated that steroid synthesis and secretion of both mineralocorticoids and glucocorticoids can be studied in adrenal slice cultures. We propose adrenal tissue slice culture as a simple *ex vivo* test system with which to examine the adrenocorticolytic activity of environmental pollutants and drugs in human and wild animal tissues.

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