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METABOLIC ACTIVATION/INACTIVATION

Species differences in 3-methylsulphonyl-DDE bioactivation by adrenocortical tissue

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Abstract The CYP11B1-activated adrenocortical toxicant 3-methylsulphonyl-DDE (3-MeSO$_2$-DDE) is proposed as a lead compound for an improved chemotherapy for adrenocortical carcinoma. We compared the binding of 3-MeSO$_2$-[^{14}C]DDE in the adrenal cortex of four rodent species; hamster, guinea pig, mouse and rat, using a precision-cut adrenal slice culture system ex vivo. Localization and quantification of the bound radioactivity were carried out using light microscopy autoradiography and radioluminography. The results revealed major species differences since 3-MeSO$_2$-[^{14}C]DDE was extensively bound to the hamster adrenal tissue while the guinea pig adrenals were devoid of binding. A high binding in mouse adrenal cortex was confirmed while binding in rat adrenal cortex was very weak. The results support previous observations that metabolic activation of 3-MeSO$_2$-DDE is highly species dependent. Since CYP11B1 could be expressed in tissues other than the adrenal cortex, final toxicological characterization should be carried out in a species that can bioactivate this compound.

Keywords 3-MeSO$_2$-DDE · Hamster · Guinea pig · CYP450 · Irreversible binding · Adrenal · Tissue-slice

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Introduction

The persistent DDT metabolite 2-(4-chloro-3-methylsulphonylphenyl)-2-(4-chlorophenyl)-1,1-dichloroethene (3-MeSO$_2$-DDE) is a potent adrenocorticolytic compound following a CYP11B1-catalysed metabolic activation and irreversible metabolite binding in the adrenal *zona fasciculata* in mice (Lund et al. 1988; Jönsson et al. 1991; Lund and Lund 1995). In contrast, only weak irreversible binding of 3-MeSO$_2$-DDE has been detected in *zona fasciculata* in rat adrenal glands investigated in cultured adrenal slices ex vivo (Lindhe et al. 2001). However, 3-MeSO$_2$-DDE is bound to human adrenal tissue following a CYP-catalysed metabolic activation ex vivo and in vitro (Jönsson and Lund 1994; Lindhe et al. 2002), supporting that 3-MeSO$_2$-DDE is a metabolism-activated adrenal toxicant also in humans.

Species differences provide a classical problem in toxicological risk assessment of drugs and chemicals, particularly when data from animal experiments are to be extrapolated to humans. Since 3-MeSO$_2$-DDE is proposed as a lead compound for developing an improved pharmacotherapy for adrenocortical carcinoma in humans (Lindhe et al. 2002), it is of interest to further examine the CYP-catalysed metabolic activation of this compound in various species.

In the present study we used a precision-cut adrenal slice culture system and applied microautoradiography and radioluminography to localize, quantify and compare the binding of 3-MeSO$_2$-[^{14}C]DDE in the adrenal cortex in four rodent species, i.e., hamster, guinea pig, mouse and rat. Based on previous results (Lindhe et al. 2001), the mouse and rat served as a positive and negative control, respectively. The results show that 3-MeSO$_2$-DDE binds extensively to the hamster adrenal

while the binding to the guinea pig adrenal was even lower than to the negative control, the rat adrenal. The results suggest a gradient (mouse > hamster > rat > guinea pig) in the magnitude of binding of 3-$MeSO_2$-DDE to the adrenocortical tissue of these rodent species.

Materials and methods

Animals

Female C57Bl mice and Sprague Dawley rats were obtained from Scanbur BK AB (Sollentuna, Sweden). Female Syrian Hamsters and Dunkin Hartley guinea pigs were from Charles River (Sulzfeld, Germany) and HB Lidköpings Kaninfarm (Lidköping, Sweden), respectively. The animals were kept on a 12 h/12 h light/dark regimen and given a standard pellet diet and tap water ad libitum. The animals were killed with CO_2 gas. The experiments were approved by the Local Ethics Committee for Animal Research (C76/2).

Chemicals

2-(4-Chloro-3-methylsulphonylphenyl)-2-(4-chloro-[^{14}C]phenyl)-1,1-dichloroethene (3-$MeSO_2$-[^{14}C]DDE; 495 MBq/mmol, purity >98%) was kindly prepared by Dr Åke Bergman, Stockholm University, Stockholm (Bergman and Wachtmeister 1977). Dimethylsulfoxide (DMSO) and agarose (Type VII, low melting temperature) were from Sigma (St Louis, MO, USA). Methacrylate Technovit 7100 was obtained from Kulzer (Wehrheim, Germany). All solvents were from Merck (Darmstadt, Germany). Liquid film NTB 2 was purchased from Kodak (Rochester, USA).

Preparation and incubation of tissue slices

The adrenals were rapidly excised from three individuals of each species, weighed and kept in ice-cold PBS buffer until being embedded in 3% agarose. Precision-cut slices (200 µm) were prepared in a Krumdieck tissue slicer (Alabama Research and Development, Munford, AL, USA) in ice-cold PBS and cultured as described previously (Krumdieck et al. 1980; Lindhe et al. 2001, 2002). 3-$MeSO_2$-[^{14}C]DDE (3.7 kBq/ml, 7.5 µM/well), dissolved in DMSO, was added to the incubation medium (DMSO did not exceed 0.5% of total volume). Slices were incubated for 24 h before being prepared for light microscopy autoradiography and radioluminography as described below. The performance of this method will henceforth be referred to as ex vivo.

Microautoradiography

After incubation, adrenal slices were fixed overnight in buffered formaldehyde (4%). The tissues were then dehydrated/extracted sequentially in 70, 90, 96%, and absolute ethanol, embedded in methacrylate and sectioned (2 µm). Embedding of cultured adrenal slices was performed in a two-step procedure as described previously (Lindhe et al. 2001). Glass slides carrying the sections were dipped in liquid NTB2 film emulsion and exposed for 6 months (4°C). The autoradiograms were developed, stained with toluidine blue, and examined with light-field and dark-field microscopy.

Radioluminography

Semi-quantification of tissue-bound radioactivity (^{14}C) was accomplished by apposing histological sections (prepared as above) to imaging plates (BAS-IP MS 2040S Fuji, Japan) (Mori and Hamaoka 1994; Motoji et al. 1995; Lindhe et al. 2001, 2002). All sections within one experiment were placed on the same imaging plate and exposed for 2 years (to enable quantification of low binding). The radioactivity in the labelled areas of the adrenal sections was recorded by reading the imaging plate in a Phosphoimager (BAS 1500 v1.8, Fuji, Japan). For semi-quantification of tissue-bound radioactivity, a Windows-based bio-imaging analyser program (MultiGauge, version 2.2, Fujifilm, Japan) was used. The labelled areas of the images (regions of interest), considered to be of adrenocortical origin, were selectively marked at 1 pixel resolution (1 pixel = 100 µm). Twenty-seven sections from three individuals of each species were evaluated. Values obtained were expressed as photo-stimulated luminescence (PSL) minus background (BG) per mm^2 of 2 µm thick tissue sections ((PSL − BG)/mm^2).

Irreversible binding in vitro

Mice, rats, guinea pigs and hamsters ($n = 5$) were killed with CO_2 and the adrenals were excised and immediately frozen at −70°C. The adrenals from each species were pooled and 300 g adrenal homogenates were prepared. A filter method by Wallin et al. (1981) was employed to determine irreversible metabolite binding. Shortly, the homogenates were incubated with 3-$MeSO_2$-[^{14}C]DDE and a NADPH-generating system for 45 min (37°C), before being transferred to glass microfibre filters (Whatman, GF/C). The filters were washed in various solvents to remove unbound substance, and the remaining non-extracted radioactivity was measured in a liquid scintillator (Tri-Carb 1900CA, Packard). The binding was expressed as pmol bound substance/mg protein.

Results

Microautoradiograms of the adrenal slices from mouse, rat, hamster and guinea pig following exposure to 3-$MeSO_2$-[^{14}C]DDE (24 h) ex vivo are shown in Fig. 1.

The mouse adrenal slices contained considerable amounts of bound substance, confined specifically to *zona fasciculata*. This was expected, since this has been reported earlier in the adrenal tissue slice culture system (Lindhe et al. 2001). Extensive binding also occurred in the hamster *zona fasciculata*, similar to the mouse adrenal. Interestingly, intensive patches of bound substance in the hamster adrenal medulla were present. No corresponding binding in the adrenal medulla was observed in any of the other species investigated. Both rat and guinea pig adrenals were devoid of specifically bound 3-$MeSO_2$-DDE, and not more than background radioactivity could be observed in these sections. The binding of radioactivity in what was considered as *zona fasciculata* of the adrenal slices was semi-quantified and the results are given in Fig. 2.

As expected, the semi-quantification by phosphoimaging shows that the binding of 3-$MeSO_2$-[^{14}C]DDE to mouse adrenal *zona fasciculata* was very intense. The binding to the hamster adrenal *zona fasciculata* was approximately as intense as that in the mouse, confirming the observations during the visual inspection. The labelling recorded in guinea pig and rat adrenal cortex was very weak.

Irreversible binding assay

In this preliminary study, binding of 3-$MeSO_2$-[^{14}C]DDE to the 300 g homogenates which could be inhibited by the potent CYP11B1-inhibitor metyrapone was considered as specific binding. The specific binding was estimated to 99.0 pmol/mg protein for the mouse, compared to only 16.0 pmol/mg protein for the rat. The newly investigated species hamster and guinea pig showed a binding of 60.6 and 0.5 pmol/mg proteins respectively. Additionally, the binding of 3-$MeSO_2$-DDE to mouse and hamster adrenal homogenates was shown to increase in a time-dependent manner (10–45 min).

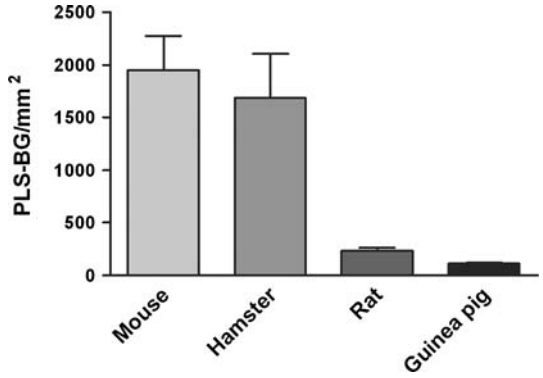

Fig. 2 Semi-quantification of the binding of 3-$MeSO_2$-[^{14}C]DDE to adrenal zona fasciculata in adrenal tissue slices from mouse, hamster, rat and guinea pig. Values obtained were expressed as photo-stimulated luminescence (PSL) minus background (BG) per mm^2 of 2 µm thick tissue sections ((PSL-BG)/mm^2). Data represent mean ± SD

Discussion

We demonstrate that 3-$MeSO_2$-DDE binds intensely to the hamster adrenal ex vivo, specifically in the cortical *zona fasciculata* and in defined patches of the medulla. In contrast, the guinea pig adrenal binding of 3-$MeSO_2$-DDE was negligible. As positive and negative controls we included

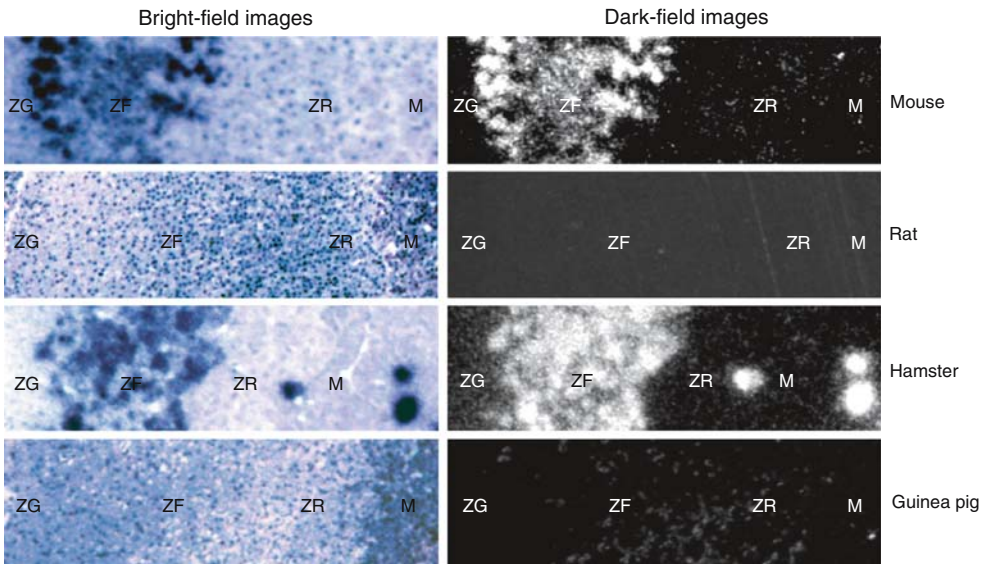

Fig. 1 Binding of 3-$MeSO_2$-[^{14}C]DDE in cultured mouse, rat, hamster and guinea pig adrenal slices. The *left side* shows light-field images and the *right side* the corresponding dark-field images of microautoradiograms stained with toluidine blue (100-fold magnification of mouse and hamster adrenals and 50-fold magnification of rat and guinea pig adrenals). *ZG* zona glomerulosa, *ZF* zona fasciculata, *ZR* zona reticularis, *M* medulla

mouse and rat adrenal slices and as expected, the binding was intense in mouse adrenal *zona fasciculata* but very poor in all zones of the rat adrenal cortex. These results confirm and clearly illustrate the striking species differences in the ability of the adrenal cortex to bioactivate and bind 3-MeSO_2-DDE. Moreover, they support the hypothesis that there might be some crucial amino acid differences at the active site of the bioactivating enzyme, CYP11B1, that determine the capacity of the enzyme to bioactivate 3-MeSO_2-DDE into a reactive, tissue-binding metabolite (Lindhe 2001).

With regard to irreversible binding and toxicity of 3-MeSO_2-DDE in the adrenal cortex, the mouse is so far the most sensitive species studied (Jönsson et al. 1991). No corresponding effects of this compound have been observed in rats in vivo (unpublished data). In the present study, the level of binding of 3-MeSO_2-DDE to hamster and mouse adrenal slices was almost equal. The binding in the hamster adrenal was localized predominantly in *zona fasciculata* in the adrenal cortex, where CYP11B1 is highly expressed in rodents (Domalik et al. 1991; Ogishima et al. 1992; Ishimura and Fujita 1997), but high radioactivity was also found in the medulla. The strong 3-MeSO_2-DDE-binding at this site was probably due to that the adrenal medulla of hamster, as well as several other species, contains single or clusters of cortical cells (Bornstein et al. 1991; Tucker 1996). Assuming that this binding was CYP11B1 dependent, these cells should consequently express a functional CYP11B1 enzyme. In baboons, cortical cells present in the medulla contain catalytically active CYP11B1 (Brown et al. 2002). The binding of 3-MeSO_2-DDE to the guinea pig adrenal slices did barely exceed background levels, and was even less pronounced than in rat adrenal slices. These findings were reflected in the preliminary study using adrenal homogenates in vitro. The rank of order of species regarding irreversible binding to the adrenal homogenates was found to be mouse > hamster > rat > guinea pig.

The ability of glucocorticoid-producing adrenal cells to activate and bind 3-MeSO_2-DDE differs greatly among different species. Table 1 compiles the knowledge about some investigated species. Covalent metabolite binding and cellular toxicity may be strongly associated (Cohen et al. 1997). It should be emphasised that even if binding of a chemical occurs, toxicity does not necessarily develop. In mice, however, the irreversible binding of 3-MeSO_2-DDE in the adrenal cortex is followed by severe toxic effects as mentioned above (Lund et al. 1988; Jönsson et al. 1991). Extrapolation of toxicity data between species should be done with caution, which becomes evident in this case. As suggested by the outline in Table 1 it seems likely, however, that predictions about the in vivo binding of 3-MeSO_2-DDE can be made by investigating the binding in vitro/ex vivo. The pronounced binding of 3-MeSO_2-DDE in mouse and chicken adrenal homogenate in vitro, is in complete agreement with the binding and subsequent toxicity that occur in vivo in these species. Based on such considerations, 3-MeSO_2-DDE should be expected to be a metabolism-activated toxicant in hamster and human adrenal cortex in vivo as well. The assumption that the human adrenal could also be sensitive is further supported by the previous finding that a normal human adrenal cortex was estimated to have about 65% of the bioactivating capacity compared to the mouse adrenal cortex using adrenal tissue slice culture technique (Lindhe 2001).

The major species differences in metabolic activation and adrenocortical toxicity of 3-MeSO_2-DDE could be due to the narrow active site of CYP11B1. The main function of CYP11B1 (11β-hydroxylase) is to catalyse the reaction of 11-deoxycorticosterone or 11-deoxycortisol into corticosterone or cortisol, and this enzyme has to our knowledge no other major endogenous substrates. To our knowledge, 3-MeSO_2-DDE is the only compound that has been conclusively demonstrated to be metabolised into a reactive, tissue-binding metabolite by CYP11B1, although several

Table 1 Compilation of data regarding irreversible binding of 3-MeSO_2-DDE to adrenal tissue in selected species

Species	Binding in vitro	Binding ex vivo	Binding in vivo	References
Mouse	+	+	+	Lund et al. (1988), Jönsson et al. (1991, 1995), Lindhe et al. (2001), present study
Hamster	+	+	×	Present study
Rat	−	(+)	−	Lindhe et al. (2001), present study, unpublished
Guinea-pig	−	−	×	Present study
Human	+	+	×	Jönsson and Lund (1994), Lindhe et al. (2001)
Seal	+	×	×	Lund (1994)
Otter	−	×	×	Jönsson et al. (1993)
Mink	−	×	×	Jönsson et al. (1993)
Chicken	+	×	+	Jönsson et al. (1994)

In vitro refers to studies with adrenal homogenates, ex vivo to studies using the adrenal tissue slice system and in vivo to whole animal studies
+, high; (+), very low but present; −, no relevant binding at the dose(s) investigated; ×, not investigated

inhibitors of this enzyme, including *o,p′*-DDD, have been described (Dörr et al. 1984; Johansson et al. 1998; Khan et al. 2003; Hermansson et al. 2007). Small mutations of amino acids in the substrate-binding region seem to be of crucial importance for the 11β-hydroxylase activity of the CYP11B1 protein regarding endogenous substrates (Böttner et al. 1996, 1998; Curnow et al. 1997). Since the enzyme has a narrow substrate specificity, disadvantageous mutations could also be critical for the ability of CYP11B1 to metabolise exogenous substrates compared to the normal enzyme. Corresponding amino acid differences at the active site of CYP11B1 could be decisive for the metabolic activation of 3-MeSO_2-DDE in different species. If such crucial amino acid differences could be identified, it would be possible to predict sensitive species.

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