CYP2A5-MEDIATED ACTIVATION AND EARLY ULTRASTRUCTURAL
CHANGES IN THE OLFACTORY MUCOSA: STUDIES ON 2,6-
DICHLOROPHENYL METHYLSULPHONE

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Abbreviations: 2,6-diClPh-MeSO₂, 2,6-dichlorophenyl-methylsulphone; CYP cytochrome P450; ER, endoplasmic reticulum; GBC, Globose basal cell; GSH, glutathione; HBC, horizontal basal cell; HMPA, hexamethylphosphoramide; NP-SH, non-protein sulphydryl; RER, rough endoplasmic reticulum; SER, Smooth endoplasmic reticulum, TEM, transmission electron microscopy, wt wild type
Abstract

2,6-Dichlorophenyl methylsulphone (2,6-diClPh-MeSO₂) is a potent olfactory toxicant reported to induce endoplasmic reticulum (ER) stress, caspase activation and extensive cell death in mice. The aim of the present study was to examine cytochrome P450 (CYP) dependent bioactivation, non-protein sulphydryl (NP-SH) levels and early ultrastructural changes in mouse olfactory mucosa following an ip injection of 2,6-diClPh-MeSO₂ (32 mg/kg). A high covalent binding of 2,6-diClPh-¹⁴C-MeSO₂ in olfactory mucosa S9-fraction was observed and the CYP2A5/CYP2G1 substrates coumarin and dichlobenil significantly decreased the binding whereas the CYP2E1 substrate chlorzoxazone had no effects. An increased bioactivation was detected in liver microsomes of mice pretreated with pyrazole, known to induce CYP2A4, 2A5, 2E1 and 2J, and addition of chlorzoxazone reduced this binding. 2,6-DiClPh-¹⁴C-MeSO₂ showed a marked covalent binding to microsomes of recombinant yeast cells expressing mouse CYP2A5 or human CYP2A6 compared to wild type. One and 4 hr after a single injection of 2,6-diClPh-MeSO₂, the NP-SH levels in the olfactory mucosa were significantly reduced compared to control whereas there was no change in the liver. Ultrastructural studies revealed that ER, mitochondria and secretory granules in nonneuronal cells were early targets 1 h after injection. We propose that lesions induced by 2,6-diClPh-MeSO₂ in the mouse olfactory mucosa were initiated by a CYP-mediated bioactivation in the Bowman’s glands and depletion of NP-SH levels, leading to disruption of ion homeostasis, organelle swelling, and cell death. The high expression of CYP2A5 in the olfactory mucosa is suggested to play a key role for the tissue-specific toxicity induced by 2,6-diClPh-MeSO₂.
The olfactory mucosa harbours a wide variety of xenobiotic-metabolizing enzymes including several cytochrome P450 (CYP) forms, the most predominant are CYP2A3/5/10/13 and CYP2G1 (Ding and Kaminsky 2003; Piras et al. 2003; Ling et al. 2004). The CYP-mediated metabolism is generally regarded as a protection mechanism and the high expression of drug metabolizing CYPs is most likely related to the clearance of odorants and other airborne chemicals. These enzymes may also protect the central nervous system against inhaled toxicants. CYP enzymes are, however also central for the metabolic activation of foreign compounds into reactive, toxic metabolites and olfactory CYPs have been suggested to play a key role in the tissue-selective toxicity of drugs and chemicals in this tissue (Gaskell 1990; Dahl and Hadley 1991; Reed 1993; Brittebo 1997; Ding and Kaminsky 2003). A decreased olfactory toxicity has been demonstrated after pretreatment with various CYP-inhibitors (Brandt et al. 1990; Genter et al. 1994; Bahrami et al. 2000b). Many olfactory toxicants and carcinogens including dichlobenil, coumarin, hexamethylphosphoramide (HMPA) and the tobacco-specific NNK [4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone] are preferentially bioactivated to reactive intermediates by the predominant expressed mouse CYP2A5 (Negishi et al. 1989; Liu et al. 1996; Thornton-Manning et al. 1997; Gu et al. 1998; Zhuo et al. 1999; Felicia et al. 2000).

2,6-Dichlorophenyl methylsulphone (2,6-diClPh-MeSO₂) (Fig. 1) is a potent olfactory toxicant in rodents, and similar 2,6-dichlorobenzene derivatives with a large, polar and strong electron-withdrawing substituent in the primary position have recently been reported as potential olfactory toxicants in mice (Brandt et al. 1990; Bahrami et al. 1999; Bahrami et al. 2000a; Carlsson et al. 2004). The 2,6-diClPh-MeSO₂-induced
olfactory toxicity is markedly reduced in animals pretreated with the broad-spectrum CYP inhibitor metyrapone and this inhibitor also abolishes the extensive bioactivation of 2,6-diClPh-MeSO₂ to a reactive, tissue-binding metabolite in rat olfactory microsomes (Bahrami et al. 2000b, Franzen et al. 2003). The role of specific CYP forms in the bioactivation of this toxicant has not been examined. The covalent binding in rat liver microsomes is very low, suggesting involvement of CYP forms preferentially expressed in the olfactory mucosa. Light microscopy autoradiography revealed that the reactive 2,6-diClPh-MeSO₂ intermediate is selectively bound to the mucus-secreting Bowman’s glands in the olfactory mucosa. The studies also demonstrated that glutathione (GSH) depletion potentiates covalent binding and toxicity in the mouse olfactory mucosa in vivo (Bahrami et al. 2000b). We have recently reported an extensive upregulation of the ER stress protein GRP78 in the Bowman’s glands following a single injection of 2,6-diClPh-MeSO₂ in mice. In addition, there is an early onset activation of the ER situated initiator caspase 12 and the downstream effector caspase 3 in these glands; both caspases are known to play a critical role in the signaling mechanisms of apoptosis (Franzen and Brittebo 2005). However, light microscopic histopathological studies revealed that the Bowman’s glands are markedly swollen and their mucus content is lost 4 hours after a single injection of 2,6-diClPh-MeSO₂, indicating necrotic cell death (Bahrami et al. 2000b; Franzen and Brittebo 2005).

The aim of the present study was to examine cytochrome P450 (CYP) dependent bioactivation, non-protein sulphydryl (NP-SH) levels and early ultrastructural changes in mouse olfactory mucosa following an ip injection of 2,6-diClPh-MeSO₂ (32 mg/kg). Covalent binding studies were conducted using mouse olfactory and liver S9-
fractions, hepatic microsomes from mice treated with the CYP-inducing agent pyrazole, known to increase the hepatic expression of CYP2A4, 2A5, 2E1 and 2J (Juvonen et al. 1987; Su et al. 1998; Xie et al. 2000), and with microsomes from recombinant yeast cells expressing mouse CYP2A5 or human CYP2A6 (Juvonen et al. 1987; Su et al. 1998; Xie et al. 2000). The effects of this olfactory toxicant on the non-protein sulphhydryl (NP-SH) levels were examined in the olfactory mucosa and liver. Finally, the early ultrastructural changes in the dorsomedial part of the olfactory region following a single ip injection of 2,6-diClPh-MeSO₂ in mice were investigated using transmission electron microscopy (TEM).
Materials and Methods

Animals

Female NMRI mice (5 weeks) were obtained from B & K Universal AB (Sollentuna, Sweden) and male DBA/2J mice (9 weeks) from M & B (Bomholtgård, Denmark). The animals were housed at 22 °C with a 12 h light/dark cycle and given a standard pellet diet (Ewos AB, Södertälje, Sweden) and tap water ad libitum. They were allowed to adapt for at least 5 days in the animal facility prior to the experiment. The studies were conducted in accordance with the guidelines of the Swedish animal welfare agency (SFS 1988:45). In addition, the studies were approved by the Local Ethics Committee for Research on Animals.

Chemicals

2,6-Dichlorophenyl methylsulphone (2,6-diClPh-MeSO₂) was prepared according to published procedures by Professor Åke Bergman and Dr Christina Larsson (Stockholm University, Stockholm) (Bergman and Wachtmeister 1987). Chemical purity (> 99.5 % pure) of the substances was assessed using gas chromatography (GC) and GC-mass spectrometry. 2,6-Dichlorophenyl-14C-methylsulphone [ 2,6-diClPh-14C-MeSO₂, spec. act. 57 mCi/mmol] was synthesised from 14C-methyl iodide and 2,6-dichlorobenzenethiol according to Bergman and Wachtmeister (1987). 5,5’-Dithio-bis(2-nitrobenzoic acid) was obtained from Sigma-Aldrich (Stockholm, Sweden). Glucose 6-phosphate, glucose 6-phosphate-dehydrogenase, NADP⁺, glutathione, coumarin and metyrapone were from Sigma-Aldrich (Stockholm, Sweden), 2,6-Dichlorobenzonitrile (dichlobenil) from Aldrich.
Chemie (Steinheim, Germany) and chlorzoxazone from Acros organics (Geel, Belgium). The epoxy embedding resin, TAAB 812, was purchased from TAAB laboratories equipment (Aldermaston, UK). Zymolyas 100-T was obtained from MP biomedicals, Burlingame, CA and yeast nitrogen base without amino acids from Sigma-Aldrich (Stockholm, Sweden).

Preparation of S9-fractions and microsomes

NMRI mice were anesthetized with gaseous carbon dioxide and exsanguinated. The olfactory mucosa and pieces of the livers were dissected, pooled, homogenized in ice-cold KCl (1.15 %) and centrifuged at 9 000 xg for 10 min. The pellets were dissolved in ice-cold 0.5 M Tris, pH 7.5, sampled for protein determination and stored at -70°C until use. DBA/2J mice were given three consecutive daily ip injections with pyrazole (200 mg/kg; dissolved in saline) or vehicle (saline). The mice were killed 24 h after the last injection and the livers were dissected, pooled and homogenized in ice-cold KCl-buffer. The homogenates were centrifuged at 12 000 xg for 20 min, the resulting supernatants were collected and centrifuged at 100 000 xg for 60 min. The pellets were dissolved in ice-cold potassium phosphate buffer, pH 7.4, sampled for protein determination and stored at -70°C until use. The protein concentration was determined by the method of Lowry et al. (1951) using bovine serum albumin as standard.

Yeast cells (*Saccharomyces cerevisiae*)

Recombinant yeast cells expressing mouse CYP2A5 or human CYP2A6 and wild type (wt) yeast cells (Pelkonen et al. 1994) were grown in cell medium containing yeast nitrogen base without amino acids, glucose and histidine. After full growth they
were centrifuged, suspended in cell medium containing 20% glycerol and stored at -70°C until use. The expression of CYP2A5 and CYP2A6 had been confirmed previously (Pelkonen et al. 1994) and were monitored by addition of the CYP2A5/6 substrate coumarin and determination of the fluorescent metabolite 7-hydroxycoumarin.

The pellets from the yeast cell culture were thawed and incubated for 90 min in 30°C in zymolyase buffer and zymolyase 100-T. After zymolyase digestion the cells were centrifuged, rinsed in zymolyase buffer, sonicated for 5 x 1 min and centrifuged at 10 000 xg for 20 min. The resulting supernatant was collected and centrifuged at 100 000 xg for 60 min. The pellets were resuspended in potassium phosphate buffer, pH 7.4 containing 20% glycerol, sampled for protein determination and stored at -70°C until use. The protein concentration was determined as described above.

Determination of covalent binding in vitro

Covalent protein binding was determined according to the method of Wallin et al. (1981) with a few modifications as follows. Incubations were made in capped tubes containing 50-100 µg protein of NMRI mouse liver and olfactory mucosa S9-fraction, pyrazole- or vehicle-pretreated DBA/2J mice liver microsomes or yeast cell microsomes, a NADPH-generating system (5 mM MgCl₂, 5 mM glucose 6-phosphate, 0.06 U glucose 6-phosphate dehydrogenase, and 1 mM NADP⁺), 100 mM Tris buffer (pH 7.5) or phosphate buffer 100 mM (pH 7.4) and 2,6-diClPh-¹⁴C-MeSO₂ (1.3-750 µM) dissolved in 2.5 µl dimethyl sulfoxide (DMSO) in a final volume of 100 µl. The effects of the broad-spectrum CYP inhibitor metyrapone (100 µM), the CYP2E1 substrate chlorzoxazone (100 µM) and the CYP2A5/CYP2G1 substrates coumarin
(100 µM) and dichlobenil (100 µM) on the covalent binding of 2,6-diClPh-^{14}C-MeSO_{2} (1.3-5.4 µM) were investigated. Results were analyzed by two-tailed, unpaired t-test and a value of P < 0.05 was considered significant. The S9-fractions were pre-incubated with the substrates/inhibitors (dissolved in DMSO, final concentration 2 %) or vehicle for 10 min at 37 °C before 2,6-diClPh-^{14}C-MeSO_{2} was added. DMSO was selected as vehicle based on a previous study on the inhibitory effects of various organic solvents on the metabolism of this compound (Franzen et al. 2003). Vials kept on ice were used as negative controls. The incubations were stopped by placing the vials on ice and transferring 80 µl of the incubation mixture to a glass microfiber filter paper (Whatman GF/C, diameter 25 mm). In order to remove unbound parent 2,6-diClPh-^{14}C-MeSO_{2} and metabolites, each filter paper was stepwise extracted in 10 ml solvent with occasional agitations, once in 95 % ethanol, twice in methanol, twice in acetone and once in n-heptane. Each extraction lasted for 10 min. The filters were then dried at room temperature. Ultima Gold™ (Packard instrument Co, USA) was added and the emittance of radioactivity was measured in a liquid scintillator analyzer (Tri-Carb 1900CA, Packard instrument Co.).

Analysis of kinetic data

Apparent K_{m} and V_{max} values for covalent binding were estimated by fitting the observed velocity (pmol/min/mg protein) versus the 2,6-diClPh-MeSO_{2} concentrations (1.3-750 µM) to the Michaelis-Menten equation. The kinetic parameters were determined for olfactory mucosa S9-fractions, pyrazole-pretreated liver microsomes and CYP2A5 yeast cell microsomes using the software program GraphPad Prism 3.0.
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Determination of NP-SH

Groups of mice (n = 6/group) were given a single ip injection of 2,6-diClPh-MeSO₂ (32 mg/kg body weight) or vehicle (corn oil, 10 ml/kg body weight). The animals were killed 1 or 4 h after injection by exposure to gaseous carbon dioxide and decapitated. The olfactory mucosa and samples of the liver were rapidly excised. Tissues from two animals were pooled and homogenized in ice-cold Na₂-EDTA. NP-SH-concentrations were then determined by the method of Sedlak and Lindsay (1968). Protein concentrations were measured as described above. Results were analyzed by two-tailed, unpaired t-test and a value of P < 0.05 was considered significant. The study was repeated once.

Electron microscopy

Mice (n = 3/group) were injected ip with 2,6-diClPh-MeSO₂ (32 mg/kg body weight) or vehicle (corn oil, 10 ml/kg body weight). One and 4 h after administration the mice were killed by exposure to gaseous carbon dioxide and quickly decapitated. The entire nasal region was dissected, gently flushed through the nasopharyngeal duct and fixed overnight in ice-cold phosphate-buffered formaldehyde/glutaraldehyde (1.5 %/1.5 %) (pH 7.4). The fixed nasal regions were decalcified with a formaldehyde/glutaraldehyde solution containing Na₂-EDTA (5.5 %) for 1 week. After decalcification a thin slice was cut at the second palatal ridge (level 3) according to Young (1981). The slices were rinsed in three changes of phosphate-buffered formaldehyde/glutaraldehyde (pH 7.4) at a total of 30 min. In addition, the slices were treated with osmium tetroxide (1 %, 2 h at 4 ℃), rinsed in PBS-buffer, dehydrated in series of ethanol, treated with acetone, and finally embedded on silicon plates in TAAB 812 resin. Sections (2 µm) were cut and stained with methylene blue.
to determine the area of examination (the dorsal meatus) in a light microscope.

Ultrathin sections (50 nm) were then cut with a diamond knife. The sections were placed on copper grids, counterstained with uranyl acetate (4 %, 30 min) and lead citrate, (0.1 M, 5 min) and examined in a Philips CM10 transmission electron microscope at 60 keV (Philips, Eindhoven, the Netherlands).

Results

Determination of covalent binding in vitro

As shown in Fig. 2A, there was a marked covalent binding of 2,6-diClPh-^{14}C-MeSO_2 to S9-fractions from mouse olfactory mucosa. The binding was concentration-dependent and the apparent $K_m$ and $V_{max}$ values were calculated to $2.5 \pm 0.25$ µM and $3.3 \pm 0.08$ pmol/min/mg protein, respectively. Two different batches of S9-fraction showed similar results. Addition of the CYP inhibitor metyrapone (100 µM) and the CYP2A5/CYP2G1 substrates dichlobenil (100 µM) or coumarin (100 µM) decreased the binding to olfactory S9-fractions to $11 \pm 0.8$, $17 \pm 1.3$, and $34 \pm 5.0$ % of the control, respectively, (metyrapone: $p < 0.001$; coumarin: $p < 0.01$; dichlobenil: $p < 0.001$) (Fig. 2B). The covalent binding of 2,6-diClPh-^{14}C-MeSO_2 to the liver S9-fraction was very low and $K_m$ and $V_{max}$ values could not be determined. Addition of the CYP2E1 substrate chlorzoxazone did not significantly change the covalent binding to olfactory S9-fractions (97 \pm 13 % of vehicle-control). In contrast, chlorzoxazone significantly decreased the covalent binding of 2,6-diClPh-^{14}C-MeSO_2 to liver microsomes from mice pretreated with the CYP-inducer pyrazole (48 \pm 10 % of the vehicle-control; $p < 0.01$).
There was a marked, concentration-dependent covalent binding of 2,6-diClPh-\(^{14}\)C-MeSO\(_2\) to liver microsomes from mice pretreated with the CYP-inducer pyrazole (Fig. 2C). The \(K_m\) and \(V_{max}\) values were estimated to 124.9 ± 10.5 \(\mu\)M and 112.6 ± 3.1 pmol/min/mg protein, respectively. The covalent binding to liver microsomes of vehicle-treated control mice was low; the predicted \(K_m\) and \(V_{max}\) values were 175.9 ± 43.5 \(\mu\)M and 40.6 ± 3.4 pmol/min/mg protein, respectively.

There was also a high concentration-dependent covalent binding of 2,6-diClPh-\(^{14}\)C-MeSO\(_2\) to microsomes from yeast cells transfected with CYP2A5 as compared to microsomes from wt yeast. The predicted \(K_m\) and \(V_{max}\) values were 163.6 ± 20.7 \(\mu\)M and 11.4 ± 0.8 pmol/min/mg protein, respectively. The covalent binding of 2,6-diClPh-\(^{14}\)C-MeSO\(_2\) to recombinant CYP2A6 yeast microsomes was less pronounced but higher than in microsomes from wt yeast (Fig. 2D).

**Determination of NP-SH**

The NP-SH levels in the olfactory mucosa of 2,6-diClPh-MeSO\(_2\)-treated mice (32 mg/kg) were reduced to 61 and 45 % of the vehicle-treated control at 1 and 4 h after a single injection (1 h: \(p < 0.05\); 4 h: \(p < 0.01\)). In contrast, no statistically significant change of the NP-SH levels was observed in the liver, 84 and 104 %, respectively, as compared to vehicle-treated controls. The level of NP-SH in vehicle-treated mice was higher in the liver than in the olfactory mucosa, 982 ± 151 and 209 ± 70 nmol NP-SH/mg protein, respectively.

**Electron microscopy**

**Vehicle-treated control mice**
The ultrastructural features of the olfactory mucosa were similar to those previously reported (Frisch 1967; Bergstrom et al. 2003). In the lamina propria, numerous Bowman’s glands, blood vessels, and axon bundles were present. The pyramidal cells of Bowman’s glands were characterized by large electron-lucent secretory granules, smooth endoplasmic reticulum (SER), rough endoplasmic reticulum (RER) and mitochondria throughout the cytoplasm (Fig. 3A, 5D). Excretory ducts of the Bowman’s glands were crossing the basement membrane passing through the neuroepithelium to the luminal surface. The duct cells close to the basement membrane contained electron-lucent secretory granules, mitochondria, SER, and RER. The apical duct cells were flat with no secretory granules. The flat duct cells had microvilli and a thin layer of electron-dense cytoplasm containing SER and RER.

The upper layer of the olfactory neuroepithelium consisted of sustentacular cells with abundant mitochondria and a microvillous border. The nuclei of the sustentacular cells were oval and contained clumped chromatin along the nuclear membrane. The cytoplasm was electron-lucent with granular features, tonofilaments, RER and SER (Fig. 5A). The middle layer was composed of olfactory neuron cell bodies with electron dense nuclei. The neurons had a columnar organization, a thin electron-lucent cytoplasm and irregular nuclei. At the luminal surface there were dendritic knobs with extended cilia (Fig. 3A). Scattered apoptotic bodies were observed. The basal layer was composed of flat electron-dense horizontal basal cells (HBC) and round lightly stained globose basal cells (GBC). The HBCs were situated in close contact with the basal lamina and the GBCs were mostly situated above the HBCs (Fig. 3A).

2,6-diClPh-MeSO2-treated mice
Bowman’s glands and excretory ducts

One h after administration there were vacuoles in intraepithelial excretory duct cells above the basement membrane (Fig. 4A). Some mitochondria in these cells were swollen and contained flocculent densities. In acinar cells in the lamina propria the secretory granule membranes were ruptured resulting in dispersed granules throughout the whole cytoplasm. The mitochondria were swollen and contained flocculent densities (Fig. 4C, 5E). The cytoplasm was vacuolated, the SER was severely swollen and both undamaged and swollen RER was present (Fig. 5E). After 4 h the size of the vacuoles had increased in the intraepithelial excretory duct cells (Fig. 3B) and most cytoplasmic organelles including mitochondria, SER and RER were severely swollen. In the lamina propria the individual acinar cells were difficult to distinguish and the radial cell organization was seldom discernible in the acini. Secretory granule content was dispersed throughout the cytoplasm (Fig. 3B, 5G). The mitochondria were severely swollen and electron-lucent with some flocculent densities. The cytoplasm was vacuolated, the SER was severely swollen and both undamaged and swollen RER were present. The acinar cell nuclei were irregular and the nuclear chromatin was aggregated and attached to the nuclear membrane (Fig. 5F and H).

Sustentacular cells

After 1 h a few mitochondria in the apical part of the sustentacular cells were swollen, less electron dense, and contained flocculent densities. Vacuoles and swelling of some SER and RER were also observed in the apical part (Fig. 4B and 5B). After 4 h most of the mitochondria in the apical part of the cells were severely swollen, less electron dense and contained flocculent densities. Both RER and SER were swollen (Fig. 5C).
The cell nuclei were first irregular (1 h) and later on regular and swollen (4 h) and the cytoplasm was more electron-lucent. Large vacuoles appeared in the apical parts. At both survival times many severely swollen mitochondria and large vacuoles occurred in the basal part of the neuroepithelium but it was not possible to elucidate if these organelles belonged to the footprocesses of the sustentacular cells or not. No apoptotic bodies were observed among these cells.

Neurons
Most neurons had an intact appearance and no marked changes were observed 1 and 4 h after administration. An expansion of the intercellular space between the columnar rows of neurons could, however, be observed. Scattered apoptotic bodies were also present.

Basal cells
After 1 h some HBCs had an electron-lucent cytoplasm (Fig. 4A). After 4 h most of the HBCs showed an electron-lucent cytoplasm and cell nuclei (Fig. 3B). Most of the GBCs had an intact appearance.

Other tissue structures
The blood vessels were dilated 1 and 4 h after administration. The axon bundles appeared intact at both survival times.
Discussion

The present study demonstrated that the potent olfactory toxicant 2,6-diClPh-MeSO₂ was bioactivated to a reactive intermediate by recombinant yeast cells expressing mouse CYP2A5, and that the CYP2A5/2G1 substrates dichlobenil and coumarin significantly reduced the bioactivation of 2,6-diClPh-MeSO₂ in mouse olfactory S9 fraction whereas the CYP2E1 substrate chlorzoxazone had no effect. Non-neuronal cells in the dorsomedial part of the mouse olfactory region are known to harbour several CYP forms such as CYP2A5 and the tissue-specific CYP2G1, which constitute more than 30% of the total CYP expression in the mouse olfactory mucosa (Ding and Kaminsky 2003; Piras et al. 2003; Ling et al. 2004). The present results also demonstrated that a single injection of a toxic dose of 2,6-diClPh-MeSO₂ induced a tissue selective depletion of olfactory NP-SH levels and early swelling of ER and mitochondria as well as rupture of secretory granules in nonneuronal cell types in the dorsomedial part of the mouse olfactory region. We propose that lesions induced by 2,6-diClPh-MeSO₂ in the mouse olfactory mucosa were initiated by a CYP-mediated bioactivation in the Bowman’s glands and depletion of NP-SH levels, leading to disruption of ion homeostasis, organelle swelling and cell death. The high expression of CYP2A5 in the olfactory mucosa is suggested to play a key role for the tissue-specific toxicity induced by 2,6-diClPh-MeSO₂.

The initial 2,6-diClPh-MeSO₂-induced damage preferentially occurs in the rodent olfactory region whereas no damage can be noted in the liver (Bahrami et al. 1999). The results of the present study confirm that the bioactivation of 2,6-diClPh-MeSO₂ is very low in the mouse liver. To investigate the role of specific CYPs in the bioactivation of 2,6-
diClPh-MeSO$_2$, covalent binding studies were conducted using hepatic microsomes from pyrazole-treated mice. Pyrazole is known to increase the hepatic expression of CYP2A4, 2A5, 2E1 and 2J (Juvonen et al. 1987; Su et al. 1998; Xie et al. 2000). There was a significantly increased level of covalent binding of 2,6-diClPh-MeSO$_2$ to hepatic microsomes of pyrazole-treated mice demonstrating that pyrazole-inducible hepatic CYPs can bioactivate this toxicant. Addition of the CYP2E1 substrate chlorzoxazone markedly decreased the covalent binding to microsomes of pyrazole-treated mice suggesting that also CYP2E1 may bioactivate 2,6-diClPh-MeSO$_2$. However, since chlorzoxazone did not significantly change the covalent binding of 2,6-diClPh-MeSO$_2$ to olfactory S9-fractions the contribution of CYP2E1 in the olfactory metabolism of 2,6-diClPh-MeSO$_2$ appears to be negligible. Furthermore, the liver normally contains relatively high levels of CYP2E1 and if this CYP form is involved in the metabolic activation and toxicity of 2,6-diClPh-MeSO$_2$ hepatic damage would be expected. However, no morphological lesion in the liver could be observed.

To further clarify the role of CYP2A5 in the bioactivation of 2,6-diClPh-MeSO$_2$, microsomes from recombinant yeast cells expressing CYP2A5 were used. The marked covalent binding detected confirmed that this enzyme bioactivates 2,6-diClPh-MeSO$_2$ to a reactive intermediate that binds covalently to protein in ER. In recombinant yeast cell microsomes expressing human CYP2A6, the bioactivation of 2,6-diClPh-MeSO$_2$ was less prominent but higher than in wt yeast microsomes. The toxicity of 2,6-diClPh-MeSO$_2$ in CYP2A6-expressing human tissue has not been examined. The marked metabolic activation demonstrated in olfactory S9-fractions was reduced by the CYP2A5/2G1 substrates dichlobenil and coumarin, supporting the involvement of at least one of these enzymes in the bioactivation of the compound.
The present study also revealed a significant depletion of NP-SH in the olfactory mucosa following a single dose of 2,6-diClPh-MeSO₂ in mice. No similar reduction was observed in liver confirming a tissue selective effect. This is in analogy with previous result showing that addition of GSH but not methyl-GSH, significantly reduces the covalent binding of 2,6-diClPh-MeSO₂ to rat olfactory microsomes (Franzen et al. 2003). The lack of effect of methyl-GSH implies that the putative electrophilic 2,6-diClPh-MeSO₂ intermediate has affinity for cysteinyl thiols.

We have recently reported that a single dose of 2,6-diClPh-MeSO₂ induces an upregulation of the ER stress protein GRP78 and activation of the initiator caspase 12 and the downstream effector caspase 3 in the Bowman’s glands of mice (Franzen and Brittebo 2005). These results imply an early activation of apoptotic pathways. The present study revealed that a single dose of 2,6-diClPh-MeSO₂ induced early and severe swelling of both ER and mitochondria in the mucus producing Bowman’s glands. The swelling of these organelles was not accompanied by an extensive formation of apoptotic bodies, although scattered cells with condensed nuclei were found. On the contrary, most of the Bowman’s gland cells rapidly became severely swollen, the cell membrane integrity was ruptured and four h after dosing the radial organization of the Bowman’s gland acini was completely lost. The extensive formation of reactive intermediates of 2,6-diClPh-MeSO₂ in ER, i.e. at the principal site of CYP localization, may be of importance for the early changes in this organelle. Notably, CYP2A5 has also been detected in the mitochondrial fraction of rodent liver tissue (Honkakoski et al. 1988). The observed ultrastructural changes are consistent with previous light-microscopic findings showing a severe swelling of Bowman’s
gland acini and suggest that the terminal phase of apoptosis was not completed. Increased levels of cytosolic Ca\textsuperscript{2+} from damaged ER stores and/or perturbation of cellular sulphhydryls will lead to insufficient ATP levels to execute the apoptotic pathway of cell death (Nicotera and Melino 2004). In addition, caspases may be inactivated by electrophilic compounds with an affinity to thiols allowing caspase-independent pathways of cell death to prevail (Finkelstein et al., 2001).

The Bowman’s glands contain a large amount of secretory granules and contribute to the composition of the mucus covering the luminal surface of the neuroepithelium. Electron microscopy revealed an early loss of secretory granules in the Bowman’s glands demonstrating that these glands are sensitive targets for 2,6-diClPh-MeSO\textsubscript{2}-induced toxicity. The secretory granule membranes were severely ruptured and the granule contents were dispersed in the cytosol. These results confirm previous light microscopic reports suggesting that loss of secretory granule contents is an early marker for a disturbed function in this cell type (Brandt et al. 1990; Bahrami et al. 2000b; Franzen and Brittebo 2005).

No early changes were observed in the olfactory neurons except for an increased intercellular space between the columnar rows of neurons following exposure to 2,6-diClPh-MeSO\textsubscript{2}. In addition, large vacuoles in the basal part of the neuroepithelium were evident, probably due to degeneration of excretory ducts of Bowman’s glands and/or foot processes of sustentacular cells.
The sustentacular cells in the neuroepithelium also showed early onset changes such as severely swollen ER and mitochondria after injection of 2,6-diClPh-MeSO₂. In addition, the apical cell membrane showed an early and extensive damage. Similar results, indicating that both the sustentacular cells and the Bowman’s glands are early target cells in the olfactory mucosa have been observed after systemic exposure to other olfactory toxicants such as 3-methylindole and a phosphodiesterase inhibitor (Pino et al. 1999; Miller and O’Bryan 2003). The earliest ultrastructural change detected in these cells is dilatation of ER.

No apoptotic bodies were observed in the sustentacular cells following exposure to 2,6-diClPh-MeSO₂. This is consistent with our previous data demonstrating that there is no early upregulation of ER stress protein or activation of caspases in the sustentacular cells and indicate that other cell death pathways occur in this cell type (Franzen and Brittebo 2005). The severe damage in the sustentacular cells may be secondary to the deleterious changes in the mucus-producing Bowman’s glands. Changed properties of the protective mucus may lead to increased exposure of the sustentacular cells to inhaled air and changes of apical cell membrane integrity may lead to perturbations of ion homeostasis and organelle swelling. There is a high expression of CYP2A5 in the apical part of the sustentacular cells (Piras et al. 2003). Notably, however, there is no accumulation of protein adducts in the sustentacular cells after injection of 2,6-diClPh-MeSO₂ in mice and rats although there are high levels of adducts in the Bowman’s glands (Bahrami et al. 2000b; Franzen et al. 2003). The lack of 2,6-diClPh-MeSO₂ adducts in the sustentacular cells suggests that 2,6-diClPh-MeSO₂ is rapidly detoxified by other xenobiotic-metabolising enzymes or that reactive intermediates in these cells are rapidly detoxified by for instance GSH.
In conclusion, the present study demonstrate that the olfactory toxicant 2,6-diClPh-MeSO$_2$ was bioactivated by recombinant yeast cells expressing mouse CYP2A5 or human CYP2A6 and that CYP2A5/2G1 substrates but not a CYP2E1 substrate reduced the bioactivation in mouse olfactory S9 fraction. The results also show that a single injection of this toxicant in mice induced a tissue selective depletion of olfactory NP-SH levels and an early onset swelling in the ER and mitochondria of the Bowman’s glands and the sustentacular cells in the olfactory epithelium. Secretory granule membranes were also identified as early targets in the Bowman’s glands. We propose that the extensive olfactory lesions induced by 2,6-diClPh-MeSO$_2$ were initiated by a CYP-mediated bioactivation and adduct formation in the Bowman’s glands accompanied by a depletion of NP-SH, resulting in perturbations of ion homeostasis, organelle swelling and cell death. The predominant expression of CYP2A5 in the mouse olfactory mucosa is suggested to play a key role for the tissue- and cell-specific toxicity induced by 2,6-diClPh-MeSO$_2$ at this site.
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References


Footnotes

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Legends for figures

Figure 1. Chemical structure of the olfactory toxicant 2,6-dichlorophenyl methylsulphone (2,6-diClPh-MeSO₂).

Figure 2. Metabolic activation in vitro of 2,6-diClPh⁻¹⁴C-MeSO₂. (A) Concentration-dependent covalent binding of 2,6-diClPh⁻¹⁴C-MeSO₂ to mouse olfactory and liver S9-fractions. (B) Effects of the CYP inhibitor metyrapone (100 µM) and the CYP2A5 substrates coumarin (100 µM) and dichlobenil (100 µM) on the covalent binding of 2,6-diClPh⁻¹⁴C-MeSO₂ to mouse olfactory S9 fractions (metyrapone: p < 0.001; coumarin: p < 0.01; dichlobenil: p < 0.001). (C) Concentration-dependent covalent binding of 2,6-diClPh⁻¹⁴C-MeSO₂ to liver microsomes from mice pre-treated with the CYP2A5-inducer pyrazole or vehicle (saline). (D) Concentration-dependent covalent binding of 2,6-diClPh⁻¹⁴C-MeSO₂ to yeast cell microsomes transfected with CYP2A5 or CYP2A6. All incubations were performed with a NADPH-generating system at 37°C for 30 min in a total volume of 100 µl. All samples were made in replicates or triplicates, mean ± SD.

Figure 3. Transmission electron microscopy showing the dorsomedial part of the olfactory region of NMRI mice given a single ip injection of (A) corn oil (vehicle) or (B) 2,6-diClPh-MeSO₂ (32 mg/kg) and killed 4 h later. In the 2,6-diClPh-MeSO₂-treated mouse (B) there are vacuoles in intraepithelial excretory duct cells above the basement membrane. In the lamina propria the individual acinar cells are difficult to distinguish and the radial cell organization is not discernible in the acini. Secretory granule content is dispersed throughout the cytoplasm. Su - s sustentacular cell, Ne -
Figure 4. Transmission electron microscopy showing the dorsomedial part of the olfactory region of NMRI mice given a single ip injection of 2,6-diClPh-MeSO$_2$ (32 mg/kg) and killed 1 h later. (A) In the intraepithelial excretory Bowman’s duct cells situated above the basement membrane there are vacuoles. (B) In the sustentacular cells a few mitochondria in the apical part are swollen, less electron dense, and contain flocculent densities. Some swelling of SER and RER and vacuoles is also observed in the apical parts of these cells. (C) In the acinar Bowman’s gland cells the secretory granule membranes are ruptured resulting in dispersed contents throughout the whole cytoplasm. The mitochondria are swollen and contain flocculent densities.

Su - sustentacular cell, Ne - neuron, Bm - Basal membrane, Bg - Bowman’s gland, Nu - Nucleus, Mi - Mitochondrion, Sg - Secretory granule. Original magnification (A) x1650, (B, C) x5200.

Figure 5. Transmission electron microscopy showing the dorsomedial part of the olfactory region of NMRI mice given a single ip injection of (A,D) corn oil (vehicle) or 2,6-diClPh-MeSO$_2$ (32 mg/kg) and killed (B, E) 1 or (C, F-H) 4 h later. Figures A-C show sustentacular cells and figures D-F and H show Bowman’s glands. (B) After 1 h a few mitochondria in the apical part of the sustentacular cells are swollen, less electron dense, and contain flocculent densities. Some swelling of SER and RER and vacuoles are also observed. (C) After 4 h most mitochondria in the apical part of sustentacular cells are severely swollen, less electron dense and contain flocculent densities. Both RER and SER are swollen. (E) After 1 h the mitochondria in the
Bowman’s glands are swollen and contain flocculent densities. The cytoplasm is vacuolated, the SER is severely swollen and both undamaged and swollen RER are present. (F) After 4 h most cytoplasmic organelles including mitochondria, SER and RER in the Bowman’s glands are severely swollen. (G) Secretory granule content is dispersed throughout the cytoplasm and the radial cell organization is not discernible in the acini. Black arrows indicate swollen, electron lucent nuclei and white arrows indicate small, electron dense nuclei. (F-H) Both swollen electron lucent nuclei and small electron dense nuclei of Bowman’s gland cells are present. Nu - nucleus, Mi - mitochondrion, ER - endoplasmic reticulum. Original magnification (A-F, H) x11500, (G) x1650.
Figure 1

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\text{CH}_3 \quad \text{O=S=O} \quad \text{Cl} \quad \text{Cl}
\]
Figure 2

A. Olfactory mucosa
- Liver

B. 

C. Pyrazole
- Vehicle

D. Mouse CYP2A5
- Human CYP2A6
- Wild type