TISSUESPECIFIC TOXICITY AND METABOLIC ACTIVATION OF 2,6-DICHLOROBENZONITRILE AND 2,6-DICHLOROTHIOBENZAMIDE IN THE OLFACTORY NASAL MUCOSA

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ABSTRACT

Following single ip injections ($\geq$ 12 mg/kg) of the herbicides 2,6-dichlorobenzonitrile (dichlobenil) or 2,6-dichlorothiobenzamide (chlorthiamid) extensive damage in the olfactory nasal mucosa of mice was observed. Necrosis of the Bowman's glands in the lamina propia was first evident whereas degeneration and necrosis of the neuroepithelium developed less rapidly. No lesions were observed in the other parts of the nasal region or in the liver. Studies with subcellular homogenates of the olfactory mucosa showed that dichlobenil was covalently bound to protein via a cytochrome P-450 dependent oxidative metabolic activation. The metabolic activation in the olfactory mucosa was highly efficient in comparison to that in the liver. Based on a similar toxicity and the ability of chlorthiamid to inhibit the covalent binding of dichlobenil in vitro, we propose that dichlobenil and chlorthiamid are metabolized to a common or to closely related cytotoxic product(s).

KEYWORDS

2,6-dichlorobenzonitrile, 2,6-dichlorothiobenzamide, olfactory mucosa, toxicity

INTRODUCTION

Dichlobenil (2,6-dichlorobenzonitrile) and its analog chlorthiamid (2,6-dichlorothiobenzamide) are broad-spectrum herbicides which are used for total weed control in non-crop situations and in aquatic weed control. Although the available toxicological documentation about dichlobenil and its analogs is limited, the toxicity has not been considered to be high (1). We have recently reported that injections of [^{14}C]-labelled dichlobenil or chlorthiamid result in high levels of irreversibly bound radioactivity in the olfactory nasal mucosa of mice (2). Subsequent studies showed that dichlobenil was highly toxic to this tissue in mice and rats whereas no lesions were observed in the other parts of the nasal cavity or in the liver (3). The tissuespecific binding and toxicity of dichlobenil in the olfactory mucosa were abolished in mice treated with metyrapone,

indicating a cytochrome P-450 mediated toxicity. In this paper we summarize results from comparative histopathological studies of dichlobenil and chlorthiamid in mice. In addition, studies on the metabolic activation of [^{14}C]-dichlobenil in the olfactory mucosa are reported.

MATERIALS AND METHODS

Dichlobenil and chlorthiamid were obtained from Aldrich-Chemie, (Steinheim, FRG). Dichlobenil-[ring^{14}C] was obtained from Sigma Chem. Co. (St Louis MD, USA). The specific activity was 16,7 mCi/mmol and the radiochemical purity 99 %.

Female C57Bl mice were given single ip injections of dichlobenil or chlorthiamid (6, 12, 25 or 50 mg/kg) dissolved in DMSO (1µl/ g b.w.). At various postinjection times (8 hours - 20 days) the mice were killed and the nasal region and pieces of the livers were dissected. The tissues were fixed, decalcified, embedded in Historesin (LKB-produkter AB, Sweden), sectioned (2 µm) and stained.

Subcellular homogenates of the olfactory mucosa and liver were prepared from female C57Bl mice (1000xg supernatant, S-1) and from female Sprague Dawley rats (microsomes). The preparations were used in incubation mixtures containing [^{14}C]-dichlobenil (18 µM) and a NADPH regenerating system. After incubation the covalent binding of radioactivity to protein was determined by repeated treatment of the protein precipitate with 1% sodium-dodecylsulphate and acetone (4).

RESULTS

Histopathology Following single ip injections of dichlobenil or chlorthiamid (12 - 50 mg/kg) there was extensive necrosis of the olfactory mucosa. The lesions were preferentially present in the dorsomedial aspects of the olfactory region, whereas the damage to the lateral aspects was less prominent. Eight hours after administration, the Bowman's glands in the lamina propria were necrotic whereas degeneration and necrosis of the neuroepithelium developed less rapidly. One day after administration the neuroepithelium in the dorsal meatus was detached from the basement membrane or had disappeared completely, leaving an denuded basal lamina. Seven - 20 days after administration the dorsomedial neuroepithelium was replaced by a respiratory-like epithelium, while in the other parts of the olfactory region the epithelium had regenerated and appeared normal. There was fibrosis of most of the lamina propria 7-20 days after administration and in the chlorthiamid-injected mice cyst-like structures appeared in the lamina propria. These structures seemed to be due to infolding and downward growth of the surface epithelium. No lesions were observed in the other parts of the nasal cavity or in the liver.

Metabolic activation of dichlobenil in vitro A marked covalent protein binding of radioactivity occurred in incubation mixtures containing subcellular homogenates from olfactory mucosa of mice or rats, [^{14}C]-dichlobenil and a NADPH-regenerating system. In rat microsomes, the apparent V_{max} for the olfactory mucosa was approximately 5 times larger than that for the liver. The apparent K_m for the olfactory mucosa was approximately 4 times lower than that for the liver. Similar results were obtained in studies with S-1 fractions from the olfactory mucosa and liver of mice.

In microsomes the covalent binding was dependent on NADPH and was decreased to 27 % of the control by the addition of metyrapone. Addition of the reducing agent sodium dithionite inhibited the binding completely. Addition of glutathione to the incubations decreased the binding to 15 % of the control. Addition of chlorthiamid to S-1 fractions of

mice decreased the binding (IC_{50}= 51 µM). By addition of superoxide dismutase in microsomal incubations the covalent binding was decreased to 36 % of the control. Addition of scavengers of O_2^- such as quercetin, alpha-tocopherol and ascorbic acid had no or limited effect on the binding. Replacement of the NADPH-regenerating system with a xantine/xantine oxidase system, known to generate O_2^- , gave insignificant covalent binding. Addition of of catalase or radical scavengers such as mannitol and DMSO to the incubations did not inhibit the covalent binding indicating that H_2O_2 or $OH\cdot$ were not involved in the activation.

DISCUSSION

The results of these studies show that dichlobenil and chlorthiamid induce an extensive damage to the dorsomedial aspects of the olfactory region following doses $\geq$ 12 mg/kg in mice. The sites of toxicity of the herbicides seem to be similar. The initial damage appears in the Bowman's glands whereas degeneration and necrosis of the olfactory neuroepithelium developes later on. As determined by microautoradiography, nonextractable dichlobenil residues were confined to the lamina propria, whereas there were no bound residues in the neuroepithelium. A previous tape-section autoradiographic study revealed a similar binding-pattern for chlorthiamid (2). Since chemically reactive metabolites preferentially become bound in the vicinty of their site of formation, we conclude that the Bowman's glands have a high ability to activate dichlobenil and chlorthiamid. We propose that the damage in the Bowman's glands is primary and results from in situ metabolic activation of the herbicides to reactive, tissuebinding species. The later developed damage in the neuroepithelium may be secondary and unrelated to covalent metabolite binding in situ.

These conclusions are further supported by experiments in vitro. In subcellular homogenates of the olfactory mucosa dichlobenil was metabolized and covalently bound to protein. The dependence of NADPH and the sensitivity to metyrapone, dithionite and glutathione favour an oxidative, cytochrome P-450 dependent activation of dichlobenil to an electrophilic intermediate. The calculated kinetic constants showed that the olfactory mucosa of mice and rats both have a higher affinity and a higher capacity to activate dichlobenil in comparison to the liver. Hence, the tissuespecific toxicity observed seems to be due to a highly efficient metabolic activation of dichlobenil in the target tissue. Since chlorthiamid inhibited the covalent binding of dichlobenil it appears as both analogs are metabolized by the same enzyme system in the olfactory mucosa. The structures of the toxic metabolites are not known at present. Both dichlobenil and chlorthiamid are metabolized to a variety of metabolites in rats, including hydroxylated and sulphurcontaining derivatives. Interestingly, all identified chlortiamide metabolites were nitriles (2). Hence, it cannot be excluded that dichlobenil and chlorthiamid are metabolized to a common toxic metabolite in the nasal mucosa.

A possible involvement of O_2^- in the metabolic activation of dichlobenil is indicated by the finding that superoxide dismutase inhibited the covalent binding. Alpha-tocopherol, quercetin and ascorbic acid which are known to react effectively with O_2^- did not, however, decrease the covalent binding. Further, there was no covalent binding of dichlobenil to olfactory microsomes when the NADPH generating system had been replaced with a xantine/xantine oxidase O_2^- generating system, indicating that unchanged dichlobenil cannot be directly activated by O_2^-. Thus further studies are needed to elucidate the possible role of O_2^- in the metabolic activation of dichlobenil.

In conclusion, the present results show that the chlorinated herbicides dichlobenil and chlorthiamid are highly potent olfactory toxicants following systemic administration in mice. Studies in vitro show that dichlobenil is metabolized in the olfactory tissue to reactive, tissue-binding intermediates. Hence, dichlobenil would be expected to be toxic also after topical application to the nasal mucosa. Since dichlobenil is a volatile fine powder, inhalation toxicity of the compound is possible, especially for those who are occupationally exposed. Also dust from chlorthiamid-containing preparations may be inhaled. The potential occupational hazard posed by these herbicides should therefore be considered.

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