PESTICIDE EXPOSURE ACCORDING TO THE CZECH TOXICOLOGICAL INFORMATION CENTRE FROM 1997 TO 2005

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Key words: Pesticides, Czech Toxicological Information Centre, Rodenticides, Intoxication, Exposure,

Introduction

Pesticides became a part of our households and may represent a danger, especially when they are ingested by children or adults1,2. Some exposures lead to life-threatening intoxications3. However little has been published about the current situation and frequency of exposures to these substances in Central Europe. The aim of this study was to describe the development and severity of exposures to pesticides, based on the calls to the Toxicological Information Centre, which serves to the Czech population of approximately 10 million.

Methods

Data taken from the Czech Toxicological Information Centre database from periods 1997−2005 were evaluated retrospectively using electronic evidence system.

Results

Overall pesticide poisonings reached 4405 in this period and accounted for 6.3 % of total calls to the Toxicological Information Centre. Forty-eight % calls concerned adults, 40 % children, and 12 % animals.

In human exposures, 59 % of calls involved men, 39 % women; in 2 % the gender was unknown. During the years, the percentage of these calls slowly decreased from 7.8 % in 1997 to 5.0 % in 2005.

Veterinary calls represented only 1.4 % of all calls, but 12 % of all calls due pesticides. The course of some intoxication was in life-threatening, with severe symptoms (4.6 %) or lethal (5.0 %).

The exact numbers of inquiries in consecutive years are given in Table I. Among insecticides, the decrease was seen especially in the group of organophosphates and carbamates. More detailed data is shown in Table II. About 79 % of all exposures occurred in the vegetation period from April to October, and only 21 % calls in the other months.

Pesticide poisoning was predominantly an accidental overdose. There were 91 % unintentional exposures, 6 % suicidal, 2.3 % occupational and 0.7 % due to aggressive behavior. Ingestion accounted for 84 %, inhalation for 13 % and skin contamination for 3 %. The symptoms of intoxication were absent in 50.5 % of subjects. About 27.5 % of patients had mild symptoms, 8 % medium, 1 % severe, in the rest of the cases the symptoms were not known at the time of the call. Death was the reason of phone call in 0.3 % of cases.

In exposures to organophosphates and carbamates, 47.4 % of the subjects had no symptoms; 27.4 % had mild symptoms, 8 % medium and 3 % severe, in other calls they were unknown. Death was known in 1.5 % calls. Only 35 % calls concerned the children.

In exposures to rodenticides 83 % patients were asymptomatic, 5 % had mild symptoms, only 1.8 % medium and 2 % severe symptoms. In other calls the symptoms were not described. About 72 % calls involved children.

In 2005, the hospitalization was found necessary only in 14.4 % of subjects. About 1/3 of them were symptomatic at the time of the call. Antidotal treatment was recommended in 1.6 % of calls only: one patient was given atropine, and the other phytonadione. In 2005 only one lethal case was registered. It concerned post-mortem consultation due to suicide with pirimicarb.

Table I
Inquiries concerning types of pesticides to the Toxicological Information Centre in the years 1997−2005

<table>
<thead>
<tr>
<th></th>
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<tbody>
<tr>
<td>Insecticides</td>
<td>224</td>
<td>291</td>
<td>335</td>
<td>189</td>
<td>198</td>
<td>217</td>
<td>192</td>
<td>183</td>
<td>153</td>
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<tr>
<td>Rodenticides</td>
<td>102</td>
<td>140</td>
<td>105</td>
<td>142</td>
<td>136</td>
<td>150</td>
<td>105</td>
<td>118</td>
<td>164</td>
</tr>
<tr>
<td>Herbicides</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>83</td>
<td>109</td>
<td>117</td>
<td>78</td>
<td>77</td>
<td>89</td>
</tr>
<tr>
<td>Other*</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>110</td>
<td>122</td>
<td>191</td>
<td>117</td>
<td>95</td>
<td>73</td>
</tr>
<tr>
<td>Total</td>
<td>326</td>
<td>431</td>
<td>440</td>
<td>524</td>
<td>565</td>
<td>675</td>
<td>492</td>
<td>473</td>
<td>479</td>
</tr>
</tbody>
</table>

* Fungicides, molluscicides, combination or unknown pesticides. -- Data not available, included into Other
Discussion

The development of inquiries to the Czech Toxico-
logical Information Centre concerning pesticides is rela-
tively favorable. It is true especially for human poisonings,
as the most dangerous products had already been replaced
by less toxic products. It can be seen in the group of or-
ganophosphates insecticides that have been substituted by
other substances, i.e., synthetic pyrethroids with low toxic-
ity for the mammals. The situation was even better in the
group of rodenticides. In the Czech Republic, solely war-
farin or superwarfarin based rodenticides are commercially
available, which explains the good course of ingestions of
these products. Comparing with the situation in the years
1988–1989 (ref.1), the percentage of suicidal attempts with
pesticides from all exposures was not different from the
recent data. However, the severity of exposures substan-
tially decreased. In the years 1988–1989, total 14 deaths
were recorded (7 after suicidal ingestions and 7 after
drinking pesticide non-intentionally from a soft-drink bot-
tle).

On the other hand, veterinary calls document a higher
danger for exposed animals, which is in agreement with the
literature4,5. One reason may be ingestion of a higher dose of the pesticide, when the animals are left alone with-
out supervision. Another explanation is the repeated ex-
sposure, in some cases even intentional, i.e. by the neighbors,
which concerns especially rodenticides poisonings. In
some instances improper use of antiparasitic preparations
caused the poisoning of cats and dogs.

Conclusions

Acute human pesticide exposure in our country is
mainly accidental and has good prognosis in general, due
to low toxicity of commercial products used. The number
of calls to the Czech Toxicological Information Centre due
to pesticides in the past 9 years is relatively stable and
shows a mild decrease since the year 2003. More import-
tant is the development of the spectrum of pesticides, in-
gested by the patients either accidentally or intentionally.
During the past years, the number of calls concerning toxic
substances, such as organophosphates and carbamates
insecticides slowly decreases. On the other hand, the num-
er of calls due to rodenticides mildly increased
however no serious sequel has been recorded. Lower number of
deaths is the most important difference from the situation
in the late 80ies, both after suicidal and non-intentional
ingestions.

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REFERENCES

2. Hrušková I., Bátorová L., Sagat T., Getlik A., Strnová J.,
3. Ritter L., Goushleff N. C. I., Arbuckle T., Cole D.,
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Key words: allergic diseases, children, environmental pollution, risk factors, heavy metals

Introduction

Rapid increase in the prevalence of allergic diseases (AD), which includes atopic eczema (AE), asthma respiratory symptoms (ARS), rhinitis allergica (RA) and food allergy (FA), in last decades and particularly in industrialized regions is, besides genetic factors, ascribed to environmental factors.

Subjects and methods

The following methods were applied: follow-up (years 1997−2003) of cohort of children (n=1997) from birth to 5 years of age (n=403) for allergic disease development in environmentally different Slovak regions, annual clinical examination of children by paediatric allergists and administration of maternal questionnaires focused on socio-economic status, life style and risk factors. Child’s peripheral blood collection for analyses of toxic metals, analysis of Pb, Cd and Hg by atomic absorption spectrometry (AAS) method, statistical evaluation of associations in EpiInfo v.6 and SPSS v.12.

Four selected Slovak regions were selected according to different predominant environmental pollutants and anthropogenic activities: industrial chemistry (CH) with the main sources of air pollution derived from chemical industry, industrial metallurgy and mining (M), agricultural (A) and rural (R) without any point source of industrial pollution.

Results

Comparison (Chi Square and Mantel-Haenszel tests) of the prevalence of AD in 5-year-old children with relation to risk factors is given in Table I. Influence of parent’s positive history of allergic diseases was confirmed. Although we did not find significant influence of concentrations of lead (median 26.5 µg L⁻¹), cadmium (median 0.128 µg L⁻¹) and mercury (median 0.42 µg L⁻¹) in 5-year-old children’s blood, we find a negative trend between the Cd levels in blood and incidence of AD in regions with different environmental characteristics (fig. 1).

Prevalence of allergic diseases, asthma respiratory symptoms and rhinitis allergica among children differed with respect to environmental characteristics of the regions. Statistically significant differences were found in the frequency of allergic diseases (fig. 2) between regions: M/R and M/CH (P=0.01). Prevalence of asthma respiratory symptoms (fig. 3) differed between regions: CH/A (P=0.05) and CH/M (P=0.01). Prevalence of allergic rhinitis (fig. 4) was different between regions: R/M (P=0.001), R/CH, R/A and CH/M (P=0.01).

Table I
Relation of risk factors and prevalence of allergic diseases among Slovak children

<table>
<thead>
<tr>
<th>Risk Factors</th>
<th>AD</th>
<th>ARS</th>
<th>RA</th>
<th>AE</th>
<th>FA</th>
</tr>
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<tbody>
<tr>
<td>Family history of AD</td>
<td>**</td>
<td>**</td>
<td>*</td>
<td></td>
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</tr>
<tr>
<td>Maternal history of AD</td>
<td>***</td>
<td>**</td>
<td>*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Paternal history of AD</td>
<td></td>
<td></td>
<td></td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td>Region type</td>
<td>**</td>
<td>*</td>
<td>***</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Type of delivery</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male gender</td>
<td>**</td>
<td>*</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mother smoked in past</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Exclusive breast</td>
<td></td>
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</tr>
</tbody>
</table>

* P ≤ 0.05; ** P ≤ 0.01; *** P ≤ 0.001

![Fig. 1. Prevalence of Allergic Diseases in 5-year-old children and Cd concentrations in blood](image-url)
Conclusions

Negative trend of AD prevalence with respect to Cd blood levels support the knowledge of immunotoxic effects of Cd. In our previous work, negative correlation was found between the concentrations of Cd and IgE levels in cord blood. Differences in AD prevalence among regions may result from different environmental exposures.

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REFERENCE

HAZARD IDENTIFICATION OF BINARY CHEMICALS MIXTURES BY QSAR TECHNIQUES

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Introduction

QSAR and QAA techniques applying knowledge of data on Tubifex tubifex test⁴ (EC50(T.t.)), R-analysis⁶ (analysis of plot of mixed EC50(T.t.) against molar fraction of a binary mixture) and partition coefficient of compounds between n-octanol and water (log P) were used to identify additivity or non-additivity of acute toxicity in mixtures of chemicals and to study their nature. The Tubifex tubifex test takes 5 minutes exposure and was verified by correlation with both log $K_{ow}$ and acute toxicity indices measured with fish and ciliates⁷.

Materials and method

Following chemicals were used: 2-nitroanisole (Aldrich, 99.9+ %), phenol (Aldrich, 99.9+ %), n-octanol (Aldrich, 99%), water (GORO AQUA 200, deionized, filtered through 0.22 mm membrane).

Determination of $K_{ow}$

The studied compound is added to a system of n-octanol and water where the volume of both phases is adjusted according to the expected value of $K_{ow}$. For each pair of tested compounds a series of binary mixtures with molar ratio of benzene 1.0, 0.9, 0.8, 0.6, 0.4, 0.2, 0.1, 0.05, and 0.0 was prepared. Each series was then tested at a sum concentration on various days and in triplicate on each day. The measurement was repeated three times with each concentration.

The system was shaken gently until equilibrium was achieved (5 to 10 hr) and then centrifuged to separate the two phases, especially if an emulsion has formed. Both phases, i.e. the n-octanol and the aqueous phase, were directly analyzed for both studied compounds using gas chromatography or liquid chromatography. The GC system used consisted of Agilent 6890N gas chromatograph with Agilent 7683 series injector and FID detector. The column was used was J&W scientific capillary column (30 m × 0.25 mm × 0.25 m). Nitrogen (99.99 %) was used as a carrying gas. Injection volume was 1 ml.

The HPLC system used consisted of two ECOM LCP 4020 pumps, a Knauer dynamic mixing chamber, and an ECOM LCD 2083 UV/VIS detector. The column used was a Chromolith® Performance RP-18e (4.6 × 100 mm) with Chromolith® Guard Cartridge RP-18e (4.6 × 5 mm) guard column (Merck, Prague, CR). Mobile phase for pump A consisted of degassed water and for pump B 4 : 1 methanol/water mixture. Injection volume was 20 µl and the flow rate was 1.2 ml min⁻¹. For analysis of samples generated in experiments was used isocratic mode 40 % A and 60 % B. The wavelength of detection was 254 nm.

Determination of $K_{mix}$

Coefficient of distribution $K_{mix}$ was determined according to⁸, using following equation:

$$K_{mix} = \frac{\sum_{i=1}^{n} \frac{Q_i}{W} \frac{Q_i}{Q_{mix}}}{\sum_{i=1}^{n} \frac{Q_i}{W} \frac{W}{Q_{mix}}}$$

where $W$ is a volume of the aqueous phase, $V$ is a volume of the octanol phase, $n$ is a number of compounds in the mixture, $Q_i$ is total amount of the compound i in the system and $K_i$ is partition coefficient of compound i. The value of $K_{mix}$ is normalized, i.e. in case of additivity the resulting $K_{mix}$ is equal 1.

Scientific graphic and analyzing software ORIGIN® was used for statistical calculations. The additivity was tested using chi-square test and t-test with Bonferonis correction.

Determination of EC50

The inhibition of the movement of oligochaeta Tubifex was measured as the effective concentration that causes 50 % of maximal response. Batches of six worms were immersed in aqueous solutions of the binary mixtures and the concentration – response curve was determined. The number of immobilized worms was counted in each batch precisely three minutes after their immersion. The measurement was repeated three times with each concentration on various days and in triplicate on each day. The reproducibility of the measurements was checked by the parallel determination of the EC50 for aqueous MnCl₂ solution⁹.

Results and discussion

Acute toxicity EC50 (normalized value) of binary mixture phenol-2-nitroanisole (fig. 1) is expressed as the inhibition of movement of oligochaeta Tubifex tubifex. The dashed line shows EC50 in case of additivity (normalized value 1). The normalization was used for purpose of a better understandable explanation of toxic effect of mixture and for mathematical modeling. Values of normalized EC50 above dashed line indicate inhibition of the toxic effect and values below dashed line indicate synergistic toxic effect.

Fig 2 represented partition coefficient $K_{ow}$ of 2-nitroanisole, fig 3 partition coefficient $K_{ow}$ of phenol and fig 4 normalized coefficient of distribution $K_{mix}$ of the
mixture phenol-2-nitroanisole, total concentration of the compounds were 0.02 mol l\(^{-1}\).
In the real life, the exposure to chemicals in mixture is more common than exposure to single compounds. Resulting activity should be different from additivity approach, widely used in studies on mixture toxicity\(^5\). The analysis indicates that the mixtures of industrial solvents phenol and 2-nitroanisole indicates clear “mixture interaction”, i.e. inhibition or potentiation depending on ratio of components in the mixture. An attempt to simulate the relationship between EC50 and molar ratio of mixture with log \(K_{ow}\) is shown.

Using of \(K_{mix}\) for QSAR modeling seems to be not fully sufficient. As is shown at fig. 4, there are no big changes of the \(K_{mix}\) for whole range of the mixture. On the other hand, the log \(K_{ow}\) of phenol and 2-nitroanisole show significant differences from log \(K_{ow}\) for both pure compounds. Especially the log \(K_{ow}\) of 2-nitroanisole could explain the acute toxicity of phenol – 2-nitro-anisole. On fig. 2 is shown decrease of \(K_{ow}\) for 2-nitro-anisole. It is suggested that lower value of \(K_{ow}\) of compound in the mixture lead to inhibition of toxicity of this mixture due to the less accumulation of 2-nitro-anisole, which is more toxic than the phenol, in an organic tissue of worms.

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REFERENCES

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THIODIGLYCOLIC ACID – INDICATOR OF METABOLIC UNEQUILIBRIUM

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Key words: thiodiglycolic acid, TDGA, metabolism, creatine, glutathione

Introduction

Thiodiglycolic, also thiodiacetic, mercaptodiacetic acid or dicarboxymethyl sulfide, S(CH₂COOH)₂, TDGA, is one of the normal products of human metabolism and it occurs at low concentrations in urine1,2. It is believed to be formed in a number of natural metabolic pathways however, the most frequently studied pathway involves the formation of TDGA from two carbon (2C) released from carcinogenic xenobiotics (e.g., vinylchloride monomer, ethylene oxide, 1,2-dichloroethane, chloroalkyl ethers1-8). These 2C units are oxidized via cytochrome P450 to ethylene oxide, 1,2-dichloroethane, chloroalkyl ethers1-8). These 2C units are oxidized via cytochrome P450 to chloroacetaldehyde, which reacts with reduced GSH3,8. These 2C units are oxidized via cytochrome P450 to chloroacetaldehyde, which reacts with reduced GSH3,8. From this point is this metabolic pathway common with formation of TDGA from glycine over glyoxylic and glycolic acids3,9. The process in presence of transaminases and vitamin B₆ continues over S-carboxymethyl-L-cysteine10,11, or to antihistamine Zyrtec (cetirizine dihydrochloride)2.

Some bacterial strains can metabolize TDGA which in turn gets oxidized to thiodiglycol sulfoxide. A significant increase is observed when the organism is exposed to the above mentioned 2C units, to the cytostatics ifosfamide and thiotepa3, or to the S-carboxymethyl-L-cysteine10,11, or to antihistamine Zyrtec (cetirizine dihydrochloride). TDGA is also one of the final products of the degradative pathway of thiodiglycol (TDG), which is produced by alkaline hydrolysis of mustard gas (bis(2-chloroethylsulphide)) (MG)12,13. In accordance with the Chemical Weapons Convention a concept of chemical detoxification and complete biodegradation of organic products by a biotechnological method using bacteria utilizing TDG was studied by Ermakova et al. and Lee et al.12-14. TDGA is one of the final products. Some bacterial strains can therefore be used for bioremediation of MG contaminated soils. A considerations of metabolic formation and fate of TDGA, structurally an analogue of MG, the microbiological studies on MG detoxification15 seem to offer interesting aspects. Therefore, TDGA was found in urine of Iranian victims of an alleged attack with MG15.

Experimental

The analysis of TDGA was carried out by the computer-controlled Eco-Tribo Polarograph using the software Polar 5.1 version for Windows on hanging mercury drop electrode (HMDE) (all Polaro-Sensors, Czech Republic). Other experiments were performed on mercury meniscus modified or polished silver solid amalgam electrodes and on solid composite electrodes to compare the results with those obtained on HMDE. The process of TDGA reduction on the mercury electrode surface was studied in detail using Elimination Voltammetry with Linear Scan11. For characterization of body composition and of pending parameters the multi-frequency impedance analyzer “In Body 3.0” was used.

TDGA was analyzed, after pre-separation from urine on a column of powdered PVC and after elution by 0.2 M perchloric acid, by the D.C. voltammetric procedure described in1,3. The common laboratory methods were used for determination of other compounds determined parallel with TDGA, which were useful for elucidation of metabolic pathways (in blood: uric acid, folates, vitamin B₁₂, cholesterol, homocysteine (HoCySH), testosterone, cortisone; in urine: creatine, creatinine, pH, total proteins content). Their corrections for specific gravity were calculated14.

Results and discussion

The natural level of TDGA in urine of healthy volunteers varies between 10 and 20 mg L⁻¹. Its abnormally increase was found in the morning urine of individuals, who suffered from certain metabolic disorders14. The TDGA levels decreased substantially during the day. The time dependences of TDGA levels can be explained as the result of fluctuations of the daily rhythm of thiolic substances14. Creatine, consumed regularly as a food supplement, increases the TDGA level in the morning urine. Creatine supplemented to groups of randomly selected individuals, in any time of day, increased excretion of TDGA rapidly within 4–8 hours following per oral intake of creatine. Normal ranges of urinary TDGA levels were usually reached within the following 1–2 hours. The supplemented creatine mostly decreased the amount of normally excreted creatinine and increased the pH value of urine in the time of maximal TDGA excretion. The amount of excreted TDGA is specific for different individuals. It can be supposed that supplemented creatine reduces its endogenous production in humans. In accordance with previous findings2,14, vitamin B₁₂, p.o., increased the TDGA level independently of creatine supple-
mentation, and did not affect the pH-value of urine. The stimulation of TDGA excretion after vitamin B_{12} application is caused by disturbance of redox equilibria induced by an increased supply of thiolic substances. Supplementation of creatine increases the input of 1C and 2C units into metabolic pathways, participated by TDGA. An increased level of TDGA in urine indicates a disturbance of redox equilibria in human body. Creatine supplementation affects metabolism of thiocompounds and excretion of some compounds into blood and urine, the metabolism of which is connected with the synthesis of creatine or with its usage in the body. Therefore, the levels of folates, vitamin B_{12}, homocysteine in blood, the levels of creatine, creatinine, TDGA, and pH in urine were followed and evaluated. The results were accomplished by data of bioimpedance measurements. According to the changes in creatine, folates and vitamin B_{12} levels before and after creatine administration in course of one month, it was possible to divide the volunteers under study into 4 groups. Each of them is characterized by typical changes in levels of the above mentioned substances. The results confirmed that creatine given as food supplement is used in human body not only for creatine phosphate formation, but that it affects the whole metabolic transformation and body constitution (mass, fat, and proteins).

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REFERENCES

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Key words: chemical carcinogenesis, Sudan I, peroxidase, chromatography, mass-spectroscopy

Introduction

Sudan I [1-(phenylazo)-2-naphthalene, C.I. Solvent Yellow 14] was used as a food colorant in several countries, but it has been recommended as unsafe, because it causes tumors in the liver or urinary bladder in rats, mice, and rabbits, and is considered a possible carcinogen and mutagen for man. Nevertheless, it is widely used to color materials such as hydrocarbon solvents, oils, fats, waxes, plastics, printing inks, and shoe and floor polishes. Sudan I has been characterized in rabbits, while the metabolism of Sudan I is not understood in humans. It is the case of peroxidase, the metabolites formed by this enzyme have, however, not been identified as yet. Therefore, the present work was undertaken to isolate two major Sudan I peroxidase-mediated metabolites and characterize them partially by mass spectroscopy.

Materials and methods

Incubation mixtures contained the following in a final volume of 70 ml: 10 mM sodium phosphate buffer (pH 8.4), 0.5 µmol horseradish peroxidase (HRP), 100 µmol Sudan I dissolved in methanol and 200 µmol H2O2. After incubation (37 °C, 20 min) the mixtures were extracted with ethyl acetate (2 × 15 ml). The extracts were evaporated, dissolved in a methanol, and separated on HPLC or TLC. Silica-gel TLC plates were developed in hexane-diethyl ether (1:3, v/v). Spots of Sudan I metabolites M1 and M2 with Rf of 2.14 and 1.93, respectively, were extracted with methanol. Alternatively, the products were separated by HPLC on a Tessek Separon Hema S 1000 (8.0 × 250 mm) C-18 column. Gradient elution (75–100 % methanol) with a flow rate of 0.3–1.5 ml min⁻¹ was used. Sudan I metabolites were detected at 215, 254, 333 and 480 nm. Two product peaks with r.t. of 32 and 46 min (peaks 1 and 2 in fig. 1) were collected. Sudan I metabolites were analyzed by mass spectroscopy. Spectra were measured using Esquire 3000 Bruker Daltonics (APCI, ESI – positive ionization).

Results and discussion

The absorption spectrum of the reaction mixture containing Sudan I and peroxidase significantly changes during the Sudan I oxidation (fig. 2). During the reaction, the absorption maximum at 480 nm (due to Sudan I) decreases
whiles the absorbance at about 340 nm increases slightly (fig. 2). Sudan I contains a free hydroxyl group in its molecule. Many phenolic compounds can serve as substrates for peroxidases, being oxidized to phenoxyl radicals which will undergo secondary reactions in dependence on their individual free radicals chemistries. This mechanism was also found by us previously for Sudan I as a substrate; peroxidase oxidizes this carcinogen, giving rise to an oxygen-centered radical (naphthoxyl radical)\textsuperscript{11,12}. The products formed during peroxidase-mediated oxidation includes BDI and C-hydroxy derivatives [6-OH-Sudan I and 4’,6-di(OH)-Sudan I]\textsuperscript{5}. But major metabolites are unstable (sensitive to light and elevated temperature)\textsuperscript{5} and their structures have not been elucidated yet. In the present paper we used two separation procedures (TLC and HPLC) to obtain individual Sudan I metabolites in purity sufficient for their partial characterization. HPLC utilizing a Tessek Separon Hema S 1000 C-18 column was originally developed here and used for purification of the two major Sudan I oxidation products (M\textsubscript{1} and M\textsubscript{2}, fig. 3). These products were characterized by their mass spectra (fig. 4).

In all cases the products decomposed during mass spectroscopy. The fragmentation peak at \textit{m}∕\textit{z} 232 in the metabolite M\textsubscript{1} indicate the presence of the Sudan I molecule without a hydroxyl group. The peak at \textit{m}∕\textit{z} 405 suggests the presence of the ion composed from hydroxylated Sudan I and another molecule of Sudan I, but without the benzene ring and nitrogen atoms (fig. 4). The fragment peak at \textit{m}∕\textit{z} 495 in the metabolite M\textsubscript{2} indicates the ion composed of two Sudan I molecules (fig. 4). Moreover, fragmentation peaks at \textit{m}∕\textit{z} 159, 172, 247, 389 (391) and 417 (fig. 4) seem to correspond to decomposition of the Sudan I dimer molecule. Nevertheless, the real structure of the both metabolites will be evaluated in detail using NMR spectroscopy. This work is under way in our laboratory.

**Conclusion**

The results of the present and previous studies\textsuperscript{9,11,12} suggest that one-electron oxidation products (radicals) are the primary intermediates in the peroxidase-mediated oxidation of Sudan I. The fate of the primary free radical depends on the environment in which it exists. We found that

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Fig. 2. Oxidation of Sudan I by the peroxidase/H\textsubscript{2}O\textsubscript{2} system. The samples (1 ml) contained 50 mM Na phosphate buffer pH 8.4, 0.2 mg horseradish peroxidase, 0.15 mM Sudan I, and 0.5 mM H\textsubscript{2}O\textsubscript{2}. The spectra were recorded sequentially at 0 min (---), 2.5 min (-----) and 10 min (-----)

Fig. 3. HPLC of Sudan I metabolites formed by peroxidase (a) and their HPLC re-chromatography (b,c)

Fig. 4. Mass spectra (APCI) of Sudan I metabolite M\textsubscript{1} (a) and M\textsubscript{2} (b)
the Sudan I reactive free radicals: (i) form additional products; (ii) react with other compounds of potential physiological interest (reaction with NADH, ascorbate)\(^3,5,11,12\), (iii) react with SH groups of glutathione (reducing Sudan I radicals with the formation of a thyl radical\(^12\)) and (iv) react with macromolecules (DNA, RNA proteins) to form potentially toxic adducts \textit{in vitro} and \textit{in vivo}\(^5,8,12\). Structure elucidation of the Sudan I-(deoxy)guanosine-adducts\(^8,11\) will be the objective of a future study.

\textit{Supported by grants 303/05/2195, MSM 0021620808 and 1M4635608802.}

\textbf{Abbreviations}

- APCI: atmospheric pressure chemical ionization
- BDI: benzendiainzonium ion
- CYP: cytochrome P450
- ESI: electrospray-ionization
- HRP: horseradish peroxidase
- TLC: thin layer chromatography

\textbf{REFERENCES}

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Key words: vinyl chloride-monomer (VCM), demonstrated chemical carcinogen, standardised mortality ratio

Introduction

The production of the most popular plastic material – polyvinyl chloride (PVC) was started in 1933 and during the past 60 years has been applied in almost every industrial activity.

According to IARC Lyon Agency monograph of 1987 VCM is classified I. group as a demonstrated carcinogen with target effects on the liver. Exposed workers showed significantly increased incidence of liver tumours, mostly angiosarcoma (ASL), insignificant mortality rise for tumours in general, in particular higher incidence lung, brain cancers, and lymphosarcoma.

Materials and methods

Data about workers employed in the vinylchloride manufacturing plant from 1974 until the end of 2003 were obtained from the factory card file. The health status study of this manufacturing plant was designed as a retrospective cohort study exploiting data on the Czech population health status provided by Institute of Health Information and Statistics of the Czech Republic (IHIS) and National Cancer Register (NCR) also administered by IHIS. Mortality and morbidity of exposed workers was compared with an external control group, which constituted total Czech population. Data on the incidence of monitored phenomena specific for sex, five-year age groups, and calendar years were used in calculations. Indirect standardization was used to control potentially confounding effects of sex and age; changes in the morbidity a mortality trends in particular calendar periods were also taken into consideration.

Results

VCM production in Czech Republic

VCM, the polymerization of which produces polyvinylchloride is in the Czech Republic produced by a single plant. At the time when this production was launched, the effects of VCM on human organism have already been known, including the chronic and acute effects and carcinogenic hazards. All available information and hygienic instructions for the staff of the district hygienic station of the Central Bohemian locality that were in charge of the plant’s hygienic supervision ensure efficient environmental health protection of the workers.

Characteristics of the production and the workplace conditions

VCM is produced in hermetically sealed columns in the open. The initial substance is ethylene and chlorine. The maximum acceptable concentration (MAC) mean is 10 mg m\(^{-3}\), MAC limit is 30 mg m\(^{-3}\) – until June 2001 and 20 mg m\(^{-3}\) afterwards. Values measured at workplaces were usually under the MAC value limits; only exceptionally they were higher but never exceeded 100 mg m\(^{-3}\).

Malignant tumours

Vinyl chloride pilot plant production was started in 1974 full run production in 1977. Since 1974 until the end of 2003, a total of 908 employees worked in the PVC production plant with an average exposure of 9.4 years (SD 9.09). There were a total of 208 women with an average exposure of 6.7 (0.1–28.8) years and 700 men with an average exposure of 9.9 (0.1–29) years. Comparison of the cohort data with the National Cancer Register revealed 44 workers (15 women and 29 men) who were diagnosed with tumour disease by the end of 2003. Twenty seven of them died (6 women and 21 men). Angiosarcoma was not diagnosed, hepatoma was diagnosed twice, carcinoma of pancreas three times, gall bladder twice, and lung and bronchi nine times (data about smoking were not available). Analysis of malignant tumours morbidity and mortality (MT) has shown that the observed MT mortality (27) is statistically significantly different from the expected one (10.7) in men – 21 observed deaths vs. 9.37 expected (SMR 1.43; 95 % CI 1.43–3.36) as well as in women – 6 observed deaths vs. 1.35 expected (SMR 4.44; 95 % CI 2.18–8.26). Similar results were found also for total mortality and MT incidence (Table I).

Discussion

Analysis results indicate statistically significantly increased deviation of the health status of observed/followed up cohort of workers exposed to vinylchloride in comparison with the Czech population health status regarding total mortality as well as tumour mortality and morbidity. We are very careful in their interpretation as
evaluating the exposure of workers is very complicated because the individual length and intensity of exposures are very different.

All employees were included into the study even those not expected to have been exposed to carcinogens or those who worked at the worksite for one month only. We did not exclude these employees from the sample due to the total sample size. We have not calculated standardized indexes for particular types of tumour diseases due to their small numbers.

Table I
Standardized cancer mortality ratios according to calendar years

<table>
<thead>
<tr>
<th>Year</th>
<th>Female Expected</th>
<th>Male Expected</th>
<th>F+M Expected</th>
<th>Female Observed</th>
<th>Male Observed</th>
<th>SMR</th>
<th>95% Cl</th>
<th>Sig.</th>
<th>SMR</th>
<th>95% Cl</th>
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<td>1980</td>
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<td>0.147</td>
<td>0.169</td>
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<td>45.26</td>
<td>4.10</td>
<td>211.02</td>
<td>0.012</td>
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<td>1981</td>
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<td>0.152</td>
<td>0.172</td>
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<td>0.254</td>
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<td>1982</td>
<td>0.028</td>
<td>0.166</td>
<td>0.194</td>
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<td>0.496</td>
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Total 1.353 9.372 10.725 6 21 4.44 2.18 8.26 0.003 2.24 1.43 3.36 0.001

REFERENCES
ONDŘEJ SLANAŘ, HELENA BUZKOVÁ, KRISTÍNA PECHANDOVÁ, JOSEF MAREK, FRANTIŠEK PERLÍK
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Key words: pharmacogenetics, single nucleotide polymorphism, thiopurine S-methyltransferase

Introduction

Azathioprine and its metabolite 6-mercaptopurine are thiopurines with the latter acting as a purine antimetabolite which can inhibit purine biosynthesis by both de novo and salvage pathways. Metabolically, 6-mercaptopurine is converted by hypoxanthine phosphoribosyltransferase to activated 6-thioguanine nucleosides. These latter intermediates are ultimately incorporated into DNA as false bases. Inactivation of 6-mercaptopurine and 6-mercaptopurine nucleosides is mediated by methylation with thiopurine S-methyltransferase (TPMT) to inactive metabolites methylmercaptopurine or methylmercaptopurine nucleosides, respectively. TPMT is well characterized cytosolic enzyme, with several functionally important genetic polymorphisms leading to decreased enzyme activity.

There is substantial amount of evidence, which shows that individual activity of TPMT is one of the major factors for wide variation in the metabolism, the drug efficacy, and mainly severe toxicity of thiopurine drugs\(^1\)\(^-\)\(^5\). The patients, who have low or no detectable enzyme activity, suffer more frequently from myelotoxicity when treated with standard doses of azathioprine. On the other hand, subjects with high activity of the enzyme could have reduced clinical effect of the treatment. Genetic basis for such a variation in TPMT activity is known only for deficient states while no corresponding factors for ultra-rapid type of TPMT metabolism were found so far\(^6\).

Methods

Two hundred and sixty-seven young healthy volunteers (161 men and 106 women, aged 18–56 years) were included in the study after obtaining their written informed consent. All subjects participating in the study were unrelated subjects of Czech nationality.

Peripheral blood samples were collected from all volunteers in 7 ml collecting tubes with ethylenediaminetetraacetic acid (EDTA). Genomic DNA was subsequently isolated by a standard phenol-chlorophorm method and stored at 4 °C until analysis.

PCR amplification was run in a MyCycler (Bio-Rad, USA) using primers as described previously and specified in Table I. Subsequent RFLP analysis produced fragments specified in Table I, allele specific PCR was run for TPMT*2. The fragments were separated in 3 % agarose gel and visualized by staining with ethidium bromide. The primers were ordered at Sigma-Aldrich (St. Louis, USA), all other components of PCR reaction mix and Top Vision agarose were purchased from Fermentas (Lithuania).

The study was approved by the Ethics Committee of the General Teaching Hospital in Prague.

Expected genotype frequencies were calculated using Hardy–Weinberg equilibrium from the observed allelic frequencies. Prevalence was compared by the chi-square test. Microsoft Excel 8.0 (Microsoft, USA) and Statgraphics Plus 3.1 (StatPoint, Inc., USA) were used for data handling.

Results

Table II shows observed genotypes and respective allelic frequencies in our study. The most frequent allele was TPMT*3A, while no subject carrying TPMT*3B allele was found. Totally 26 (9.7 %) subjects were found to be heterozygous carriers of one of the variant alleles and 1 (0.4 %) volunteer was homozygous poor metabolizer. The distribution of genotypes was similar to the predicted numbers.

<table>
<thead>
<tr>
<th>Polymorphism</th>
<th>Primer name - sequence</th>
<th>Restriction enzyme</th>
<th>Fragment size</th>
</tr>
</thead>
<tbody>
<tr>
<td>G460A</td>
<td>P460F 5'-ATAACAGAGTGAGGGAGGCTGC</td>
<td>Acc I</td>
<td>293/207</td>
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<tr>
<td></td>
<td>P460R 5'-CTAGAACCAGAAAAGTTAG</td>
<td></td>
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<tr>
<td>A719G</td>
<td>P719R 5'-CTGATTGAGATTACAGTGGTGCACAC</td>
<td>Mwo I</td>
<td>365/267</td>
</tr>
<tr>
<td></td>
<td>P719F 5'-CAGGCTTTTAGCATATTTTCATCCCTC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>G238C</td>
<td>P2W 5'-GTATATTTTATGCAAGTGGTGCG</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>P2M 5'-GTATATTTTATGCAAGTGGTGCG</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>P2C 5'-TAAATAGGAACCATCGGACAC</td>
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</tbody>
</table>
Discussion

Similarly to our findings, variant allele TPMT*3A was reported as the most prevalent deficient allele in other Caucasian populations, whereas TPMT*3C is the most frequent variant in Asian populations. A good correlation between high concentrations of activated 6-thioguanine nucleosides and severe forms of bone marrow suppression in TPMT homozygous poor metabolizers treated with usual doses of azathioprine has been well established. Some studies revealed similar pattern of relationship between high levels of 6-thioguanine nucleosides and clinical effects in heterozygous intermediate metabolizers, but other authors did not report such findings. Although there exists such a discrepancy between the data a screening for TPMT deficient patients prior to beginning of azathioprine therapy seems to be cost effective approach. Our data provide necessary basis, for transferring the observed individual, social and economic consequences of TPMT deficiency from other Caucasians into the Czech population.

Conclusions

Our results show similar distribution of the most frequent variant TPMT alleles in comparison to other Caucasian populations. Therefore the consequences of azathioprine therapy arising from TPMT deficiency can be expected similar proportions as described in some other European countries. These results also provide basal information for future pharmacogenetic and pharmacoeconomic studies in the Czech population.

This study has been supported by grants No. IGA MZ ČR NR9094, and MSM0021620849.

REFERENCES


Table II
Number of subjects with specific TPMT genotypes and respective allelic frequencies

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<thead>
<tr>
<th>Polymorphism</th>
<th>Observed genotypes</th>
<th>Alelic frequencies [%]</th>
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</thead>
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<tr>
<td></td>
<td>w/w w/v v/v</td>
<td>w v</td>
</tr>
<tr>
<td>TPMT*2</td>
<td>266 1 0</td>
<td>99.82 0.18</td>
</tr>
<tr>
<td>TPMT*3A</td>
<td>244 22 1</td>
<td>95.52 4.48</td>
</tr>
<tr>
<td>TPMT*3B</td>
<td>267 0 0</td>
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<tr>
<td>TPMT*3C</td>
<td>264 3 0</td>
<td>99.44 0.56</td>
</tr>
</tbody>
</table>

w – wild-type allele, v – variant allele
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Keywords: Rockwool, oxidative DNA damage, DNA repair; antioxidative enzymes

Introduction

Oxidative damage of biomolecules (DNA, proteins, lipids) caused by free radicals is involved in the pathogenesis of different diseases such as cancer, atherosclerosis, inflammation, etc.1 Cells respond to toxic levels of reactive oxygen species (ROS) by activating a diverse arrays of protective responses. This includes a complex range of enzymatic antioxidants such as catalase (CAT), glutathione peroxidase (GPX), superoxide dismutase (SOD), glutathione-S-transferase (GST) and non-enzymatic antioxidants such as glutathione, tocopherols and carotenoids2. Oxidative repair mechanisms, includes DNA repair, protein and lipid repair, is degradation pathways3.

The adverse effect that arises from exposure to asbestos has stimulated an extensive research into the development of substitute materials. One such substitute is rockwool, made from natural basic rock material, which is used mainly as insulation for thermal, acoustic and fire protection of roofs, walls and floors. However, little is known about the health effects of these fibres. The potentially harmful effects of all types of respirable fibres are at present one of the most important fields of interest in industrial hygiene4.

Subjects and methods

In order to study the effect of rockwool exposure on oxidative DNA damage and lipid peroxidation, an epidemiological study was conducted in a rockwool factory in Slovakia.

To examine the interactions between fibre- and cigarette smoke- induced effects, the cohorts examined included non-smokers as well as individuals smoking different numbers of cigarettes. Altogether 141 subjects were investigated (21 to 58 years old), 43 controls (20 men, 23 women and 27 non-smokers, 16 smokers) and 98 exposed (75 men, 23 women and 61 non-smokers, 37 smokers). The rock wool exposure lasted at least 5 years.

All subjects contributed a single blood donation in autumn. A urine sample was used for measurement of cotinine to determine smoking status. Blood was collected by venipuncture from fasted subjects, using an anticoagulant as EDTA used for isolation of lymphocytes and erythrocytes. The samples of plasma, lymphocytes and lymphocyte extracts were stored on ice until assayed or were frozen at −80 °C for various biochemical measurements.

Measurement of DNA damage and repair

The comet assay (single cell alkaline gel electrophoresis)5 was applied to freshly isolated lymphocytes (Lymphoprep, Nycomed, Oslo, Norway) for measurement of DNA strand breaks, oxidized bases and DNA alkylation6,7. The assay for OGG1 activity5 was used to measure the ability of a cell-free lymphocyte extract to incise substrate DNA containing 8-oxoGua6,7.

Measurement of activities of antioxidant enzymes

Erythrocytes were washed three times with isotonic saline. Isolated erythrocytes were used for measurement of activities of antioxidant enzymes. The activity of glutathione peroxidase (GPx) was determined indirectly by oxidation of NADPH to NADP+ measured by the kinetic method according to Paglia and Valentine8. Catalase (CAT) was measured spectrophotometrically by a modified method of Cavarocchi et al.9 and glutathione-S-transferase (GST) by a kinetic method according to Habig et al10. The activity of superoxide dismutase (SOD) was estimated by a commercial test (Randox lab, Ltd., U.K.).

The index of the combined non-enzymatic antioxidant capacity of plasma (FRAP) was measured spectrophotometrically according to Benzie and Strain11 ferric to ferrous ion reduction at low pH causes formation of a coloured ferrous–tripyridyltriazine complex.

Ceruloplasmin oxidase activity in plasma was assayed with the use of o-dianisidine dihydrochloride according to the method of Schosinsky et al.12 Plasma vitamin C was detected by HPLC13. Lipid peroxidation was determined by the levels of malondialdehyde (MDA) using a modified HPLC method in plasma14.

Results and discussion

Rockwool exposure induced elevation of DNA strand breaks in the lymphocytes of investigated subjects (P=...
When analysed according to sex and smoking habit, this effect was apparent only in the group of non-smokers. DNA strand breaks were higher in exposed subjects compared to controls ($P=0.004$). The effect of occupational exposure to rockwool on oxidative DNA damage and repair was already published by Dusinska et. al.15: DNA repair of oxidative damage in lymphocytes of exposed subjects was higher in group of men ($P=0.02$) compared to women, and controls ($P=0.07$).

We found higher MDA levels in the group of all exposed workers ($P=0.025$) and in exposed non-smokers ($p=0.003$) possibly as the consequence of significantly suppressed activity of CPL-oxidase ($P=0.02$, 0.016 respectively) and CAT in these groups ($P=0.04$, 0.012 respectively). The activity of GST was affected by exposure to rockwool; the GST levels were significantly lower in the all exposed subjects ($P=0.04$), in the exposed non-smokers ($P=0.03$), and exposed men ($P=0.007$). Concentration of vitamin C in plasma and the FRAP were not affected by the rockwool exposure.

There was a significant negative correlation between the activity of GPX and MDA in the whole group ($P=0.007$) and in the exposed group, and between CAT activity and MDA in the all subjects ($P=0.009$). We found inverse correlations between activities of several antioxidant enzymes (GPX, GST, and CAT) and DNA damage. GST activity correlated inversely with oxidised purines measured as Endonuclease sensitive sites almost in all subgroups: in all subjects ($P=0.002$), in exposed (0.014), men (0.038), women (0.004), and exposed men (0.041). We found a significant negative correlations between DNA repair and GPX in all subjects as well as in control men ($P=0.03$, 0.028 respectively) and CAT in all control subjects and control men ($P=0.019$, 0.009 respectively). Interestingly we found the positive correlations between DNA repair and MDA in all subjects and in all exposed ($P=0.008$, 0.026 respectively).

### Conclusion

The presented results indicate that rockwool exposure induces an increase in the oxidative damage of biomolecules especially in the group of male non-smokers; however, the optimal levels of antioxidants could have a protective effect.

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**REFERENCES**

Key words: Taxus, Taxine, TLC, intoxication

Introduction

The toxicity of yew (Taxus baccata) has been known since antiquity. It was believed that "a tree of death" it endangered lives of those who stayed in its shade for a longer time. Yew was used in homicide, suicide, and as an abortive. All parts of yew, except for the red epicarp of the berries, are toxic. The toxicity of yew is attributed to taxin, a mixture of several pseudoalkaloids, in which the evidently dominant components are taxin B and isotaxin B. These taxin alkaloids are cardiotoxic. Their effect can be determined as soon as 1/2 hour after ingestion. Death after 2 and 1/2 hours after ingestion of two handfuls of yew leaves was reported.

Description of toxicological proofs of yew poisoning is not very frequent in literature. There is a description of intoxication evidence by thin layer chromatography, of the evidence achieved by chromatogram similarities between yew extract and extract of stomach content (GC/FID), of the finding of 3,5-dimethoxyphenol by gas chromatography (3,5-dimethoxyphenol is considered as a marker of yew ingestion) and the evidence of taxin B and isotaxin B determined in biological material by liquid chromatography with mass detector.

In the following paragraphs the authors describe a case of lethal intoxication caused by the decoction of yew leaves, and the proof of it by thin layer chromatography (TLC).

Case report

A 28-year-old man was found dead in the family house. For toxicological analysis the police supplied a brown liquid (pH 9) found in a bottle and a glass in the living room. Forensic autopsy stated the following: acute catarhal esophagitis, acute gastritis, a mash of plant material in the stomach and duodenum (in dry form 3.6 g), epicranial, pleural and epipatic pete-chiae, brain swelling, haemorrhagical swelling of the lungs, acute venostasis of the parenchymatous organs, and liquid status of the blood. The following was supplied for toxicological analy-
sis: blood, urine, samples of the brain and lung tissue, samples of the liver and kidney tissue and stomach and duodenum content (pH 8). No blood alcohol was detected. Nor any volatile toxic compounds in the brain and lung tissue were found. Toxicological analysis using GC/MS detected carboxy-tetrahydrocannabinol, caffeine and nicotine in the blood. Morphological and anatomical evaluation of the mash plant material found in the stomach content detected the presence of Taxus baccata leaves (evaluation performed by RNDr. Dagmar Nová).

Materials and methods

Material used

Mixture of taxin B and isotaxin B prepared in the Institute of Legal Medicine, University Hospital Münster. Chromatographic plates Kieselgel 60 F254 Merck, Fast Blue B reagent (FBB), Ehrlich reagent, Drägendorff reagent, ethanol 96 %, organic solvents used in extraction and preparation of mobile phases were p.a. purity, ammonium hydroxide solution 26 % p.a., hydrochloric acid conc. p.a.

Extraction of comparative plant material

100 ml of 3.7 % hydrochloric acid was added to 50 g of Taxus baccata leaves, the mixture was homogenized and macerated for 1 hour, then filtered. The filtrate was extracted twice with 250 ml diethyl ether and then alkalized to pH 10 with the solution of sodium hydroxid and extracted twice with 250 ml diethyl ether and 250 ml chloroform. The acidic and basic extracts were evaporated and the evaporates dissolved in 1.5 ml ethanol.

Extraction of section material

Samples of liver and kidney tissue (50 g) were homogenized, 24 hours soaked in acetone and ethanol mixture (450 ml, 7:3) and then the macerate was filtered and evaporated to 50 ml. The stomach content was diluted with distilled water and filtered. Urine, the macerate of the liver and kidney tissue, the filtrate of the stomach content and the found brown liquid were acidified with hydrochloric acid to pH 3 and extracted 2 times with 250 ml diethyl ether. The water fractions were subsequently alkalinized to pH 10 with the solution of sodium hydroxid and extracted with 250 ml diethyl ether. The acidic and basic extracts were evaporated and the evaporates dissolved in 1.5 ml ethanol. Further, urine was hydrolyzed and after neutralization extracted with 250 ml diethyl ether.
Mobile phase

A ethyl acetate : ethanol : ammonium hydroxide solution 26 % = -36:2:2
B chloroform : ethanol : ammonium hydroxide solution 26 % = 38:2:1 / paper

Abbreviations

RfA retention factor in mobile phase A
RfB retention factor in mobile phase B

Results

Fast Blue B detection

Some substances from acidic extract of yew show a very prominent colour reaction with the FBB reagent. In acidic extracts of stomach content, urine and the found brown liquid FBB reagent proved a presence of substances of corresponding character with substances contained in the extract of Taxus baccata. A bright orange-red spot in the position RfA=0.8; RfB=0.4 was particularly persuasive. A presence of substances contained in Taxus baccata extract was not determined in the liver and kidney tissue nor in the urine hydrolysate. FBB reagent showed no colour reaction with taxin B / isotaxin B standard.

Ehrlich reagent detection

With the Ehrlich reagent some substances from acidic and basic extracts of yew show a prominent colour reaction after heating the chromatographic plate. In acidic and basic extracts of the stomach content and urine Ehrlich reagent proved a presence of substances of corresponding character with substances contained in the extract of Taxus baccata. Particularly persuasive was a pink spot on the chromatogram in the acidic extract in position RfA=0.8; RfB=0.4. After heating the plate, intensified spots of blue-violet colour showed on the chromatogram of basic extract of yew, and two of them also appeared in the basic extract of stomach content (RfA/A2=0.7/0.6; RfB/B2=0.5/0.4). A presence of substances contained in Taxus baccata extract was not determined in the liver and kidney tissue nor in the urine hydrolysate. Ehrlich reagent showed no colour reaction with taxin B / isotaxin B standard.

Drägendorff reagent detection

Substances contained in basic and acidic extracts of yew react with the Drägendorff reagent. Some spots on the chromatogram of basic extract of yew correspond with spots on the chromatogram of basic extract of stomach content. Because of multiple spots the chromatogram is less clear. A presence of substances contained in Taxus baccata extract was not determined in the liver and kidney tissue nor in the urine.

REFERENCES

P55 REDUCTIVE ACTIVATION OF ENVIRONMENTAL POLLUTANTS 3-NITROBENZANTHROINE AND 2-NITROBENZANTHROINE

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Key words: 3-nitrobenzanthrone, 2-nitrobenzanthrone, reduction, NAD(P)H:quinone oxidoreductase, HPLC

Introduction

3-Nitrobenzanthrone (3-nitro-7H-benzanthracen-7-one, 3-ABA, fig. 1), occurs in diesel exhaust and in airborne particulate matter1. 3-NBA might originate both from incomplete combustion of fossil fuels and from reaction of the parent aromatic hydrocarbon with nitrogen oxides in the atmosphere. 3-NBA can spontaneously isomerize to 2-nitrobenzanthrone (2-nitro-7H-benzanthracen-7-one, 2-NBA), which can become more than 70-fold airborne particulate matter1. 3-NBA might originate both from incomplete combustion of fossil fuels and from reaction of the parent aromatic hydrocarbon with nitrogen oxides in the atmosphere. 3-NBA can spontaneously isomerize to 2-nitrobenzanthrone (2-nitro-7H-benzanthracen-7-one, 2-NBA), which can become more than 70-fold higher in concentration in ambient air1,2. 3-Aminobenzanthrone (3-ABA, fig. 1), suggested to be the main reductive metabolite of 3-NBA, has been found in urine samples of salt mine workers occupationally exposed to diesel emissions3, demonstrating that human exposure to 3-NBA in diesel emissions can be significant and is detectable. 3-NBA is carcinogenic in rats, causing lung tumours after intratracheal instillation4. It is also an exceptionally potent mutagen5. Its genotoxicity has been further documented by the detection of specific DNA adducts formed in vitro as well as in vivo in rodents6,7. Most of the metabolic activation of 3-NBA in vitro is attributable to human and rat cytosolic NAD(P)H:quinone oxidoreductase (NQO1), while human N-O-acetyltransferase (NAT), NAT2, followed by NAT1, sulfotransferase (SULT), SULT1A1 and, to a lesser extent, SULT1A2 are the major phase II enzymes activating 3-NBA (ref. 3). Microsomal NADPH:cytochrome P450 (CYP) reductase is also effective in the activation of 3-NBA (ref. 6), but in a model organism, mice, 3-NBA is predominately activated by cytochrome nitroreductases such as NQO1 rather than microsomal NADPH:CYP reductase7 (fig. 1).

While 3-ABA was suggested to be the main reductive metabolite of 3-NBA (ref. 3), the reactions of 3-NBA to 3-ABA by enzymatic systems in vitro and in vivo has not been investigated as yet. Reductive metabolism of 3-NBA is, therefore, investigated in this work. In addition, reduction of 3-NBA is compared with that of its isomer 2-NBA.

Materials and methods

3-NBA, 2-NBA, 3-ABA and N-OH-ABA were synthesized as described1,2,8. Incubations with human recombiant NQO1 or rabbit NADPH:CYP reductase, in a final volume of 500 µl, consisted of 50 mM Tris-HCl buffer (pH 7.4), containing 0.4 % Tween 20, 1 mM NADPH, from 5 to 50 µM 3-NBA or 2-NBA (in DMSO) and from 10 to 100 µg ml⁻¹ of NQO1 or NADPH:CYP reductase. In incubations testing the time-dependent formation of 3-NBA-DNA adducts mediated by human recombinant NQO1 (20 µg ml⁻¹), incubation times varied between 15 and 60 minutes and 20 mM 3-NBA were used. The incubation mixtures were extracted with ethyl acetate (2 × 1 ml) and 5 µl of 1 mM phenacetine was added as an internal standard. The extracts were evaporated to dryness;
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Fig. 2. HPLC of incubations of 3-NBA with NQO1 (a) and NADPH:cytochrome P450 reductase (b)

Fig. 3. HPLC of incubations of 2-NBA with NQO1 (a) and NADPH:cytochrome P450 reductase (b)

Fig. 4. Time-dependence of 3-NBA reduction by NQO1

residues dissolved in 30 µl of methanol, and subjected to RP-HPLC to evaluate the amounts of products of 3-NBA and 2-NBA reduction. HPLC was under isocratic conditions of 70 % methanol, with a flow rate of 0.6 ml min⁻¹. The modeling of the binding of 3-NBA to the active site of NQO1 was performed with the program AutoDock 3.0.3 and Sybyl 6.6.5 (Tripos GmbH, Germany) by the procedure described.

Results and discussion

Human NQO1 metabolizes 3-NBA to one major product identified as its reductive metabolite found in urine samples of humans exposed to diesel emissions, 3-ABA. Both compounds were separated by HPLC as two distinguish peaks (fig. 2). NQO1 is the more effective enzyme to form this product than NADPH:CYP reductase (fig. 2a,b). In contrast to 3-NBA, no 2-NBA metabolites were generated by both enzymatic systems, which indicate that this compound is a much worse substrate (if any) for these enzymes than 3-NBA (fig. 3a,b). The metabolism of 3-NBA by human recombinant NQO1 was studied in detail. We investigated the time-dependence of 3-NBA reduction (fig. 4), dependence of converted 3-NBA and produced 3-ABA on NQO1 (fig. 5) and 3-NBA concentrations (fig. 6). A time-dependent decrease in 3-NBA concentrations in incubations correlated with an increase in 3-ABA formation (fig. 4). Reduction of 3-NBA to 3-ABA by human NQO1 in the presence of NADPH exhibits the Michaelis-Menten kinetics (data not shown). The values of Michaelis constant $K_m$ of human NQO1, measured for 3-NBA reduction and 3-ABA production, were 10.3 and 7.6 µM, respectively.

In order to examine the molecular basis of the potent reduction of 3-NBA by human NQO1, the binding of 3-NBA to the active centre of NQO1 was modeled (fig. 7). The model structures for the human NQO1-3-NBA-complex were calculated. It is evident that 3-NBA fits well into the active site of human and rat NQO1, being bound near the isoalloxazine ring of the flavin prosthetic group of the enzyme. This allows an electron transfer during the reduction of 3-NBA. The value of the apparent dissociation constant for the human NQO1-3-NBA-complex was calculated to be 0.26 µM, respectively.

Fig. 5. Dependence of 3-NBA reduction by NQO1 on the enzyme concentration
Conclusion

The results demonstrate for the first time that 3-NBA is reduced by NQO1 and NADPH:CYP reductase to 3-ABA. The results also explain a strong mutagenicity and carcinogenicity of 3-NBA in contrast to 2-NBA. Much higher mutagenicity and carcinogenicity of 3-NBA evolve from its potential to be easily reduced to reactive species binding to DNA.

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Abbreviations

CYP cytochrome P450
NQO1 NAD(P)H:quinone oxidoreductase
NATs N-O-acetyltransferases
SULTs sulfotransferases
N-OH-ABA N-hydroxy-3-aminobenzanthrone

REFERENCES