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BIOLOGICAL MONITORING OF EXPOSURE TO ETHYLENE OXIDE IN THE STERILIZATION UNITS

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Key words: ethylene oxide, biological monitoring, globin adduct

Introduction

Ethylene oxide (EO, fig. 1), an important industrial intermediate, is also used as a sterilizing agent for heat-sensitive medical equipment and consumables. Due to its electrophilic nature, EO is a directly alkylating agent producing covalent adducts with biological macromolecules including DNA. EO is a rodent carcinogen and was classified as a human carcinogen[1] or a suspect human carcinogen[2]. To reduce the carcinogenic risk in occupationally exposed workers, exposure to EO should be minimized and controlled by adequate measurements. EO has an olfactory detection threshold of ca. 1200 mg m^{-3}, therefore, it cannot be perceived at concentrations normally present at workplaces. Recent studies have shown that exposure to 1.8 mg m^{-3} (1 ppm) EO would not significantly contribute to human cancer risk[3]. Thus, this value was adopted as a threshold limit value by ACGIH[2] or as a technical exposure limit by DFG[4]. The permissible exposure limit (PEL) for EO in the Czech Republic is 1 mg m^{-3}. Currently, the major source of exposure to EO are sterilization units in hospitals or in specialized facilities. These exposures, which are typically intermittent and highly variable, are commonly assessed by environmental monitoring of airborne EO. Biological monitoring based on the determination of EO adducts with blood protein globin is a valuable alternative. The major advantage of globin adducts is their long-term persistence in the organism, allowing molecular dosimetry of EO over the whole lifespan of erythrocytes, i.e., 4 months in humans. The current methodology is based on determination of the EO adduct with *N*-terminal valine of globin (2-hydroxyethylvaline, HEV) using modified Edman degradation procedure[5]. This includes isolation of globin followed by conversion of HEV using pentafluorophenylisothiocyanate reagent to 1-(2-hydroxyethyl)-5-isopropyl-3--pentafluorophenyl-2-thiohydantoin (HE-PFPTH) (fig. 1), which is determined by GC/MS.

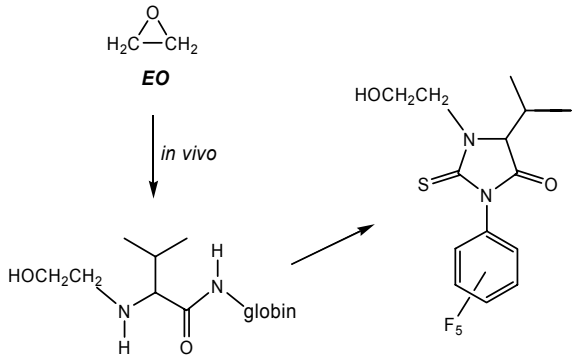

Fig. 1. **Structure of compounds dealt with in the study**

The HEV adduct is present in human globin at physiological levels. In non-smokers with no known exposure to EO, the background levels are about 20–60 pmol g^{-1} globin[6–9]. Smoking increases the HEV level by ca. 10 pmol g^{-1} per cigarette per day[7–9]. From large-scale epidemiological studies it was estimated that long-term occupational exposure to 1 mg m^{-3} EO (8 h/day, 5 days/week, >20 weeks) would result in HEV levels of ca. 3.8 nmol g^{-1} globin[7].

To our knowledge, biomonitoring of EO has not been carried out in the Czech Republic so far. In this study, we adapted the above methodical approach to assess occupational exposure to EO in 3 sterilization units in the South Morava region.

Experimental

Chemicals

N-2-Hydroxyethylvaline-leucine-anilide (calibration standard) and *N*-2-ethoxyethylvaline-alanine-anilide (internal standard) were bought from Bachem. Pentafluorophenylisothiocyanate reagent was from Fluka. Formamide for molecular biology (Merck) was distilled before use. Other chemicals were from various sources.

Subjects

Twenty six subjects (20 men, 6 women; 12 smokers) participated in the study. They were engaged in various operations in two plants sterilizing medical equipment (A, n=14; B, n=8), or performed sterilization of collection items in a museum (C, n=4). Ten subjects (6 men, 4 women, all non-smokers) with no occupational exposures to EO were used as controls. Whole blood was taken from

each person by venipuncture over heparin. Blood samples were stored at 4 °C and processed within 3 days after sampling.

Analytical procedure

Globin was isolated from the whole blood (10 ml) using a standard procedure that included separation, washing and hemolysis of the erythrocytes, followed by precipitation of globin with 2 % hydrochloric acid in acetone, washing and drying[10]. Determination of HEV in globin was carried out as described in[6]. Briefly, globin (100 mg) was dissolved in formamide (3 ml), then 1 M-NaOH (30 μl), solution of the internal standard *N*-2-ethoxyethylvaline-leucine-anilide (10 nmol) in MeOH (100 μl), and pentafluorophenylisothiocyanate (10 μl) were added. The samples were incubated overnight and then extracted with diethylether. The extract was evaporated to dryness and the residue dissolved in toluene. The samples were washed with water and 0.1 M-Na_2CO_3. Then, acetylating mixture (acetonitrile/acetic anhydride/triethylamine 3:1:1, 100 μl) was added and the sample was left at room temperature for 15 min. The sample was brought to dryness and dissolved in toluene (50 μl); 1 μl was analyzed by GC/MS.

GC/MS conditions

The analyses were performed using a DSQ GC/MS instrument (Thermo) on a capillary column DB-5ms (J&W), 30 m × 0.25 mm, film thickness 0.25 μm. Injector was set in a splitless mode (0.75 min). Flow rate of the carrier gas helium: 1.2 ml min^{-1}. Temperature of the column: 90 °C for 0.75 min, 15 °C min^{-1} to 190 °C, then 30 °C min^{-1} to 310 °C. Temperatures of the injector, transfer line, and ion source: 270 °C, 275 °C, and 200 °C, respectively. Detector was operated in the electron impact (EI) ionization mode (70 eV).

Quantitation

Quantitation was based on the ratio of peak areas of acetyl-HE-PFPTH and 2-ethoxyethyl-PFPTH (EOE-PFPTH), both monitored at *m/z* 308. For construction of calibration curve, samples of control globin (100 mg) were spiked with the calibration standard *N*-2-hydroxyethylvaline-leucine-anilide (0–2 nmol). The internal standard *N*-2--ethoxyethylvaline-alanine-anilide (10 nmol) was added and the samples were processed as described above. (On applying the modified Edman degradation procedure, the calibration and internal standards are converted to HE-PFPTH and EOE-PFPTH, respectively.)

Results and discussion

In preliminary GC/MS analyses, performance of the HE-PFPTH determination depended markedly on the cleanliness of the chromatographic system. In routine measurements on a „casual" column, progressive peak widening and tailing was observed. Introduction of an additional derivatization step, namely, conversion of HE-PFPTH to its acetyl derivative, amended the analyses dramatically. The symmetrical peak of acetyl-HE-PFPTH eluted at a retention time close to that of HE-PFPTH and afforded mass spectrum with identical major fragments (*m/z* 308, 350). Acetylation had no effect on the peak of the internal standard. Retention times of acetyl-HE-PFPTH and EOE-PFPTH were 9.20 and 8.78 min, respectively.

The calibration curve was linear over the whole range (0–2 nmol calibration standard) but only the range of 0−0.2 nmol was used for calculation of the calibration curve, y = 0.1581x + 0.0017 (y = peak area ratio of acetyl-HE-PFPTH vs. EOE-PFPTH; x = amount of calibration standard in nmol per sample). The intercept 0.0017 was attributed to the background level of HEV in control globin, employed as a matrix in the calibration samples. This background value, assessed as 0.0017/0.1581 = 0.11 nmol g^{-1}, was then used to correct the calculated HEV levels in unknown samples.

The HEV levels found in the potentially exposed workers and in controls are shown in Table I and fig. 2. Subjects with significantly elevated adduct levels were identified in each of the three facilities A-C, with few values approaching the biological exposure limit of

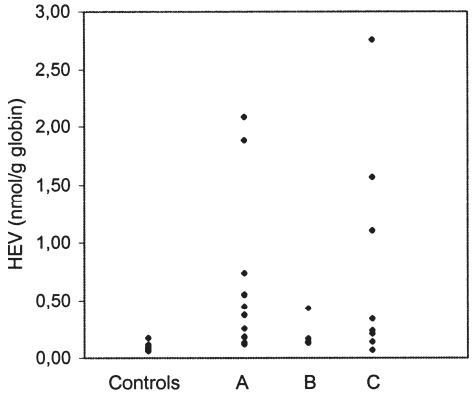

Fig. 2. **Individual values of HEV level in the examined subjects**

Table I
HEV levels in controls and subjects potentially exposed to EO in facilities A-C

	HEV levels [nmol g^{-1} globin]			
	Controls	A	B	C
n	10	14	4	8
Geom. mean	0.09	0.41	0.19	0.41
Max. value	0.17	2.08	0.43	2.76

3.8 nmol g^{-1} globin. All the highest-exposed subjects (>1 nmol g^{-1} globin) were operators of the sterilization chambers. The EO exposures in other workers not directly involved in operating the chambers (stockroom workers, repairmen, cleaning personnel, etc.) resulted in HEV levels <1 nmol g^{-1} globin.

The current study didn´t include simultaneous determination of EO in the workplace air. In the past years, short-term samplings (5−30 min) during the critical operations in the same plants revealed EO concentrations up to 80 mg m^{-3}. However, the personnel is usually wearing protective masks during such operations. The biological monitoring approach presented here indicates that despite the episodes of massive EO leaks, the average long-term exposures to EO were most likely below the level of 1 mg m^{-3}.

In conclusion, operations in the sterilization units using EO are associated with exposures that do not exceed recommended limits but still warrant further attention and support taking suitable protective measures at the workplace.

The study was supported by the Internal Grant Agency of the Czech Ministry of Health, project NJ 7387-3, and by the Regional Institute of Public Health in Brno, project TÚ 2.16.

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KINETICS OF ELLIPTICINE OXIDATION BY CYTOCHROMES P450 1A1 AND 1A2 RECONSTITUTED WITH NADPH:CYTOCHROME P450 REDUCTASE

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Key words: ellipticine, cytochromes P450, NADPH:cytochrome P450 reductase, kinetics

Introduction

Ellipticine (5,11-dimethyl-6*H*-pyrido[4,3-*b*]carbazole, fig. 1), an alkaloid isolated from *Apocyanaceae* plants, exhibits significant antitumor and anti-HIV activities[1,2]. The main reason for the interest in ellipticine and its derivatives for clinical purposes is their high efficiencies against several types of cancer, their rather limited toxic side effects, and their complete lack of hematological toxicity[1]. Nevertheless, ellipticine is a potent mutagen (for review see[1,2]). The prevalent mechanisms of ellipticine antitumor, mutagenic and cytotoxic activities were suggested to be (*i*) intercalation into DNA (ref.[2,3]) and (*ii*) inhibition of DNA topoisomerase II activity (for review see[1,2]). We demonstrated that ellipticine covalently binds to DNA after being enzymatically activated with cytochrome P450 (CYP) enzymes or peroxidases[2–6]. Human and rat CYPs of 1A and 3A subfamilies are the predominant enzymes catalyzing oxidation of ellipticine either to metabolites that are excreted from organisms (7-hydroxy- and 9-hydroxyellipticine) or form DNA adducts (13-hydroxy- and 12-hydroxyellipticine, the latter formed also spontaneously from another ellipticine metabolite ellipticine N^2-oxide by the Polonowski rearrangement)[2–5] (fig. 1). Of the peroxidases, human cyclooxygenase (COX)-2, ovine COX-1, bovine lactoperoxidases, human myeloperoxidase and horseradish peroxidase efficiently generated ellipticine-derived DNA adducts[6]. The same DNA adducts formed by ellipticine were also detected in V79 Chinese hamster lung fibroblasts transfected with human CYP3A4, 1A1 and 1A2 (ref.[7]), in human breast adenocarcinoma MCF-7 cells[8], leukemia HL-60 and CCRF-CEM cells[9] and *in vivo* in rats exposed to this anticancer drug[4,10]. On the basis of these data, ellipticine might be considered a drug, whose pharmacological efficiency and/or genotoxic side effects are dependent on its enzymatic activation in target tissues. Here, we investigated the efficiency of purified CYP1A1 and 1A2 enzymes reconstituted with NADPH:CYP reductase to oxidize ellipticine. In addition, kinetics of ellipticine oxidation by these enzymes was evaluated.

Materials and methods

The rat CYP1A1, rabbit CYP1A2 and rabbit NADPH:CYP reductase were isolated as described[2]. Incubation mixtures used to study ellipticine metabolism contained the following in a final volume of 500 μl: 100 mM potassium phosphate buffer (pH 7.4), 1 mM $NADP^+$, 10 mM D-glucose 6-phosphate, 1 U ml^{-1} D-glucose 6-phosphate dehydrogenase, 0.01–1 μM CYPs reconstituted with NADPH:CYP reductase in liposomes and 10 μM ellipticine dissolved in 10 μl DMSO. The enzyme reconstitution was performed as described[2,3], but different ratios of

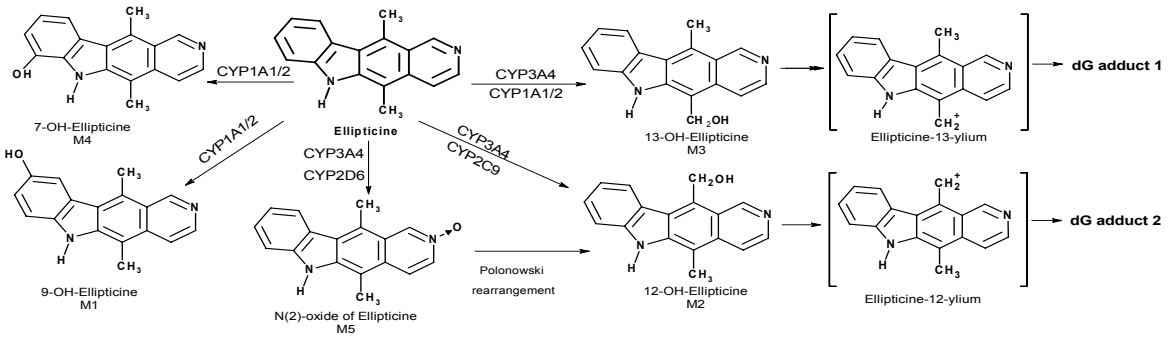

Fig. 1. **Metabolism of ellipticine by human CYPs showing the characterized metabolites found to form DNA adducts**

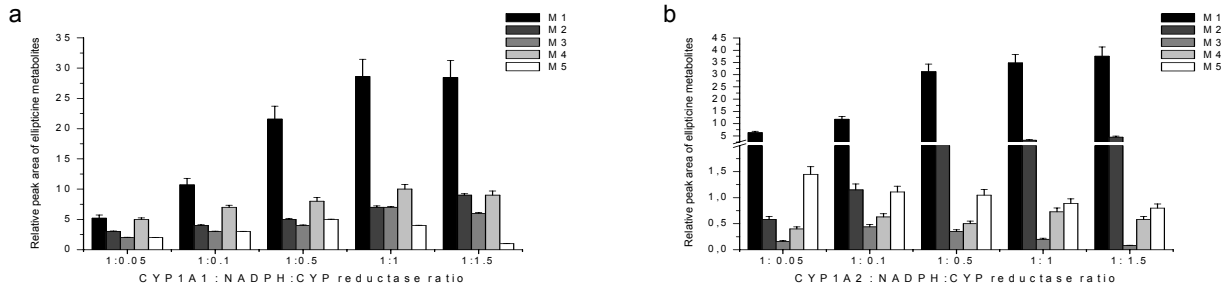

Fig. 2. **The effect of NADPH:CYP reductase on ellipticine oxidation by CYP1A1 (a) and CYP1A2 (b)**

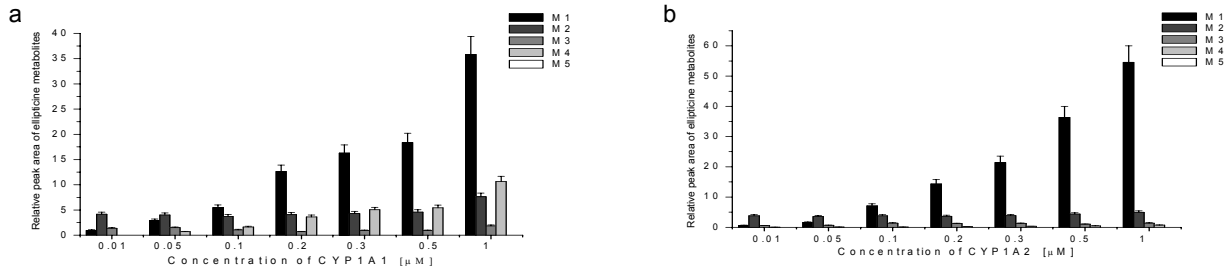

Fig. 3. **The effect of different concentrations of CYP1A1 (a) and CYP1A2 (b) on ellipticine oxidation**

CYP:reductase were utilized (fig. 2 and 3). After incubation (37 °C, 20 min), the reaction was stopped by adding ethylacetate. Thereafter, 5 μl of 1 mM phenacetine in methanol was added as an internal standard and the ellipticine metabolites were extracted twice with ethylacetate (2 × 1 ml). The extracts were evaporated under nitrogen and dissolved in 20 μl of methanol. The ellipticine metabolites were separated by HPLC as described[5]. Five ellipticine metabolites identified previously as 9-hydroxy-, 12-hydroxy-, 13-hydroxy-, 7-hydroxyellipticine and ellipticine N^2-oxide, were eluted at the retention times of 6.3, 6.9, 7.8, 8.5 and 11.2 min, respectively[5].

Results and discussion

The CYP1A1 and 1A2 enzymes reconstituted with NADPH:CYP reductase oxidized ellipticine to five metabolites: 9-hydroxy-, 12-hydroxy-, 13-hydroxy-, 7-hydroxyellipticine and ellipticine N^2-oxide (figs. 2,3), found previously to be formed by human, rat and rabbit hepatic microsomes[5]. The 9-hydroxy- and 7-hydroxyellipticine are the major ellipticine metabolites formed in the enzyme reconstituted systems containing CYP1A1 and 1A2. These results correspond to those found by us in rat, rabbit and human hepatic microsomes utilizing inducers and inhibitors of CYPs[11]. Efficiencies of CYP1A1/2 enzymes reconstituted with its reductase to oxidize ellipticine depends on the CYP:reductase ratios in the reconstitution systems. An

Table I
Kinetics parameters of ellipticine oxidation by CYP1A1 (A) and CYP1A2 (B)

CYP1A1 (A)				CYP1A2 (B)			
Ellipticine metabolites	*n*	V_{max} [min^{-1}]	K_m [μM]	Ellipticine metabolites	*n*	V_{max} [min^{-1}]	K_m [μM]
M1	1.01	1.34	0.10	M1	1.01	1.00	0.23
M2	0.91	0.08	0.82	M2	1.03	0.06	1.63
M3	0.89	0.06	3.50	M3	1.08	0.10	14.00
M4	0.98	0.22	0.51	M4	1.03	0.02	6.93

increase in the NADPH:CYP reductase content in the reconstitution systems resulted in an increase of ellipticine oxidation up to the value of the CYP:reductase ratio of 1:0.5, with negligible or low, insignificant, changes in their efficiencies up to ratios of 1:1 for CYP1A1 and 1:1.5 for CYP1A2 (fig. 2). The ratio of CYP1A1/2:reductase of 1:0.5 was used for evaluation of kinetics of ellipticine oxidation by these CYP enzymes.

An increase in the concentration of CYP1A1 and 1A2 in incubations results in an increase in formation of ellipticine metabolites, predominantly in generation of 9-hydroxyellipticine and 7-hydroxyellipticine, being linear up to CYP concentrations of 0.2 μM (fig. 3). The Michaelis-Menten kinetics was found for oxidation of ellipticine by CYP1A1 and 1A2 (data not shown). The values of Michaelis constant (K_m) and maximum velocity (V_{max}) are shown in Table I.

Conclusion

The results demonstrate that the system of purified CYP1A1 and 1A2 reconstituted with NADPH:CYP reductase oxidizes ellipticine mainly to 9-hydroxy- and 7-hydroxyellipticine, which reflects the situation of the ellipticine oxidation in human, rat and rabbit hepatic microsomes.

Supported by the GACR (grant 203/06/0329) and the Czech Ministry of Education (grants MSM0021620808 and 1M4635608802 - Centrum of targeted therapeutics).

Abbreviations

CYP cytochrome P450
K_m Michaelis constant
n Hill coefficient
V_{max} maximum velocity

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P38
THE EFFECT OF VEGETARIAN DIET ON IMMUNE RESPONSE

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Key words: vegetarian diet, immunity, elderly

Introduction

Several papers document the health benefits of vegetarian dietary practices and the lower incidence of chronic disease, especially heart disease, in vegetarians[1]. Much of the data are derived from investigations in vegetarians, most of them consume a lacto-ovo vegetarian diet. Strict vegetarian or vegan diets, which exclude all foods of animal origin, are increasingly being adopted. The adequacy and nutritional effect of diets based entirely on plant foods is still under investigation. There is a lack of data on possible immunomodulatory effect of diet in vegetarian population. In our study, health status of younger and elderly women habitually consuming a vegetarian diet was evaluated by hematological and immunological measures in comparison with a non-vegetarian group.

Subjects and methods

Study population: Our study population consists of the group of 105 younger women (20–30 years old), (52 non-vegetarians and 53 vegetarians) and group of 69 elderly women (60–70 years old), (35 non-vegetarians and 34 vegetarians).

Design of study: Cross-sectional comparison of vegetarians and age/sex-matched omnivores.

Immunological methods: Phagocytic activity was measured after engulfment of bacteria Staphylococcus aureus marked with fluorescein isotiocyanate (FITC) and respiratory burst of neutrophils was evaluated using hydroxyethidine (HE), simultaneously.

Lymphocyte proliferation was measured by [^{3}H] thymidine incorporation after incubation and stimulation with concanavalin A, phytohemaglutinin, pokeweed mitogen and CD3 antigen.

Natural killer cytolytic activity was determined in peripheral blood mononuclear cells using K562 as target cells. K562 were labeled with fluorescein isothyocyanate (FITC) and propidium iodide (PI) and evaluated by flow cytometry.

Hematological parameters were measured by Sysmex K4500.

Statistical analysis was done using SPSS (SPSS Co., USA). Differences between groups were analyzed using Student T-test.

Results and discussion

The objective of this study was to assess the immune status of vegetarians compared with non-vegetarians in younger and elderly women population. Our results indicate that vegetarian life style resulted in suppression of innate and acquired cellular immune functions. Significantly lower phagocytosis of monocytes and granulocytes was found in elderly vegetarian vs. non-vegetarian population (P<0.05, P<0.001). Similar effect of diet was observed as decreased phagocytic activity of granulocytes in

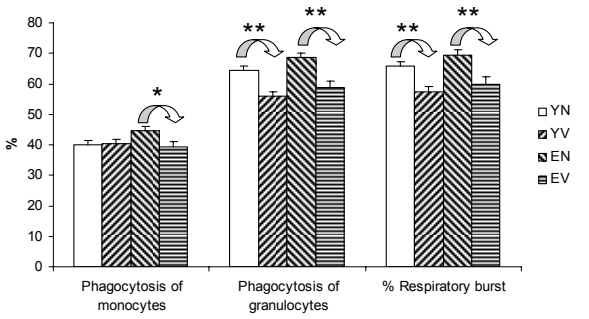

Fig. 1. **Phagocytosis of monocytes, granulocytes and percentage of respiratory burst in vegetarian and non-vegetarian population.** Statistical significance: *P<0.05, **P<0.01. YN – younger non-vegetarians, YV – younger vegetarians, EN – elderly non-vegetarians, EV – elderly vegetarians

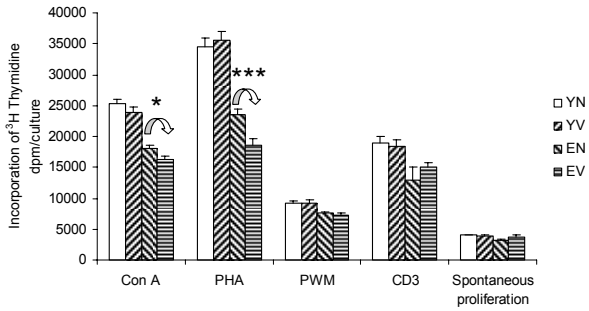

Fig. 2. **Proliferative response of T-lymphocytes and T-dependent B-lymphocytes** and spontaneous proliferation (dpm-disintegrations per minute) in vegetarian and non-vegetarian population (Con A – concanavalin A, PHA – phytohemaglutinin, PWM – pokweed mitogen, CD3 – CD3 antigen). Statistical significance: *P<0.05, ***P<0.001. YN – younger non-vegetarians, YV – younger vegetarians, EN – elderly non-vegetarians, EV – elderly vegetarians

younger vegetarians. Regardless the age, also respiratory burst of phagocytic cells was significantly decreased in vegetarians (fig. 1).

Decreased immune response was found in our elderly vegetarian population also in lymphocyte activity. Suppressive effect of vegetarian diet was markedly expressed in proliferative capacity of T-lymphocytes derived from elderly vegetarians and *in vitro* stimulated with Con A and PHA (fig. 2). Number of lymphocytes was also depressed. Our data are in contrary with findings of Richter et al. who observed identical proliferation of mononuclear cells after stimulation with interleukin-2 (IL-2) and phytohemagglutinin when compared immune function in people consuming two different diets (lacto-ovo vegetarian and meat-rich western diet)[2]. We can hypothesize, that our younger vegetarian population similarly to Richter population of male athletes is able to compensate the gap in specific nutrients. In elderly population suppression became more significant.

Table I
Hematological parameters in younger and elderly non-vegetarians and vegetarians.
Statistical significance: **P*<0.05, ***P*<0.01, ****P*<0.001

		Non-vegetarians	Vegetarians	Sign.
Leukocytes,	younger	6.64	5.84	**
$\cdot 10^3\ \mu l^{-1}$	elderly	7.33	5.25	*
Erythrocytes,	younger	4.58	4.55	
$\cdot 10^6\ \mu l^{-1}$	elderly	4.98	4.65	***
Hemoglobin,	younger	13.9	13.42	*
g dl^{-1}	elderly	15.12	14.33	**
Hematocrit,	younger	44.78	43.34	*
%	elderly	48.58	46.06	**
Platelets,	younger	205.54	209.94	
$\cdot 10^3\ \mu l^{-1}$	elderly	177.97	178.74	
Neutrophils,	younger	3.57	2.93	***
$\cdot 10^3\ \mu l^{-1}$	elderly	3.38	2.96	
Lymphocytes,	younger	2.3	2.23	
$\cdot 10^3\ \mu l^{-1}$	elderly	2.1	1.73	***
Monocytes,	younger	0.56	0.5	*
$\cdot 10^3\ \mu l^{-1}$	elderly	0.52	0.45	
Eosinophils,	younger	0.17	0.16	
$\cdot 10^3\ \mu l^{-1}$	elderly	0.17	0.17	
Basophils,	younger	0.04	0.03	***
$\cdot 10^3\ \mu l^{-1}$	elderly	0.05	0.03	**

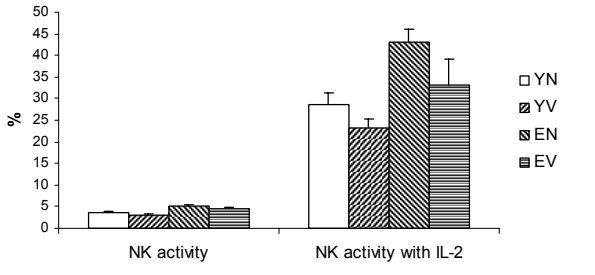

Fig. 3. **Natural killer cell activity (%) (without and with interleukin-2)**. YN – younger non-vegetarians, YV – younger vegetarians, EN – elderly non-vegetarians, EV – elderly vegetarians

Population of natural killer cells seems to be more stable towards different intake of nutrients after eating of various diets. No dramatic differences in natural killer cells activity were found between our young and elderly women populations eating different diets (fig. 3). Even in vegans with substantially lower dietary fat intake, natural killer cell activity did not differ from that of non-vegetarians[1]. Moreover, some published papers described significantly higher cytotoxic activity in vegetarians compared with their omnivorous controls[3].

In our study, elderly and younger population eating vegetarian diet had significant changes in red and white blood cells. All vegetarians had significantly suppressed levels of hemoglobin and hematocrit, older population had also decreased number of erythrocytes. Furthermore, all vegetarians had significantly lower white blood cell count and count of basophiles. Younger population had depressed number of neutrophils and monocytes (Table I). All hematological parameters were in physiological range for our laboratory. Our findings are in agreement with data of Pongstaporn and Bunyaratavej[4] who also found significant alterations in erythrocytes and leukocytes in vegetarian population. Published studies in vegans showed that some blood parameters such as lymphocyte count and mean cell volume was found even changed when compared with lacto- or lacto-ovo vegetarians. Some authors assume that lower lymphocyte and platelet count are accompanied by metabolic evidence that indicated vitamin B12 deficiency[5].

Conclusion

The objective of this pilot study was to assess the immune status of vegetarians compared with non-vegetarians. Analysis of immune functions of vegetarians displayed significantly lower phagocytosis of monocytes and granulocytes and respiratory burst of phagocytic cells. In elderly vegetarians, significantly suppressed proliferative response of T-lymphocytes in response to mitogens was found. Natural killer cell activity in population of vegetarian did not differ from non-vegetarian. Results showed lower count of white blood cells, red blood cells, hemoglobin, hematocrit, as well as neutrophils, lymphocytes, monocytes and basophils in vegetarians compared with non-vegetarians. In conclusion, our data indicate that vegetarian diet might have possible impact on immune response.

We would like to express our gratitude to Viera Vachalková, Helena Turazová and Edita Mrvíková. This

work was supported by Slovak Research and Development Agency APVV-21-013202 and APVV-21-017704.

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P39

SUPPRESSION OF OXIDATIVE BURST OF NEUTROPHILS WITH METHOTREXATE IN RAT ADJUVANT ARTHRITIS

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Key words: neutrophil, arthritis, methotrexate, chemiluminescence, reactive oxygen species

Introduction

Neutrophils are the most abundant cells present in the joints of rheumatoid arthritis (RA) producing toxic products such as proteases and reactive oxygen species (ROS). In RA patients the apoptosis of neutrophils is delayed and this may lead to increased tissue damage and failure of the inflammation to resolve[1]. Activated neutrophils emit light from unstable high-energy ROS produced by the plasma-membrane-associated NADPH oxidase and metabolized by cytoplasmic and granule enzymes. The limitation of neutrophil-mediated damage relies in part on the modification of the capacity to generate chemical damage. The light signal from activated neutrophils can be enhanced in the presence of luminol[2]. Low-dose methotrexate (MTX) has become the first-line therapy for treatment of RA. Different mechanisms of action have been suggested for its action[3]. In this study we investigated the effect of low-dose MTX on oxidative burst of blood phagocytes, predominantly neutrophils, in a model of rat adjuvant arthritis (AA).

Methods and materials

Male rats (150–170 g) induced with AA by means of *Mycobacterium butyricum* in Freund's complete adjuvans[4] were pretreated orally with MTX 0.5 mg kg^{-1} two times a week during 28 days. Blood was taken by tail venepuncture in the amount of 10 µl and diluted 200 times with Tyrode solution. Luminol-enhanced emission of light stimulated by singlet oxygen, dependent on both the superoxide and metabolisation of myeloperoxidase released from primary granules. Chemiluminescence (CL) of both spontaneous and phorbol-myristate-acetate (PMA)-stimulated blood was measured in samples containing 50 µl of diluted blood, luminol (250 µmol L^{-1}), horseradish peroxidase (8 U ml^{-1}), PMA and phosphate buffer in 50 µl aliquots. CL was measured in Luminometer Immunotech during 60 min. Neutrophil count was measured in whole blood in Coulter counter by a standard procedure. Mean integral values of CL curves over 3600 s were evaluated. Statistical significance of differences between means was established by Student's t-test and *P* values below 0.05 were considered statistically significant.

Results and discussion

The development of AA in rats was accompanied with an increase in blood neutrophil count when compared with control animals from $0.92.10^4$ to $2.29.10^4$ cells μl^{-1}, as demonstrated in fig. 1. MTX did not alter the absolute neutrophil count in blood, most probably because neutrophils in RA have delayed apoptosis and this inflammatory disease is considered anti-apoptotic. Recent evidence using animal models has shown that neutrophils play a key role in the initiation and progression of AA (ref.[1]). Free radicals, including ROS, play a crucial role in the inflammatory and immunity processes involved in RA (ref.[1,5]). Spontaneous CL of the blood of MTX-treated animals and untreated AA-animals was significantly increased on day 7 of AA development and did not further increase until day 28.

Blood stimulation with PMA resulted in an increase of CL in both groups of animals (MTX-treated, untreated) in a dose-dependent manner.

Fig. 2 demonstrates the reactivity of neutrophils (phagocytes) on day 7,14, 21 and 28 of AA development. Spontaneous CL increased significantly both in the adjuvant arthritis and methotrexate-treated group on day 7 and this difference persisted until day 28 of investigation. MTX significantly decreased CL of whole blood stimulated with PMA (0.005 µmol L^{-1}) by 35 %, as compared with the untreated AA group. A significant inhibitory ef-

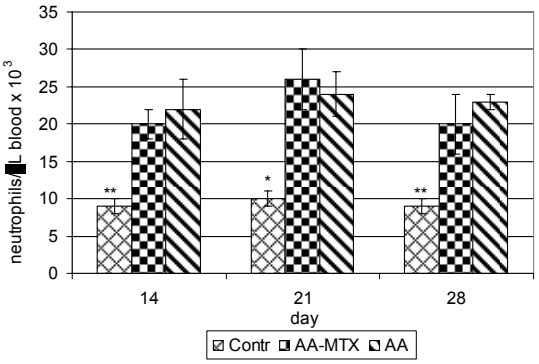

Fig. 1. **Neutrophil count in whole blood;** the values represent the mean from 6 animals as measured in 1 µl of whole blood. Contr – untreated animals, AA – adjuvant arthritis, AA-MTX – animals with adjuvant arthritis pretreated with methotrexate. Mean ± SEM, * $P<0.05$, ** $P< 0.01$ (vs AA)

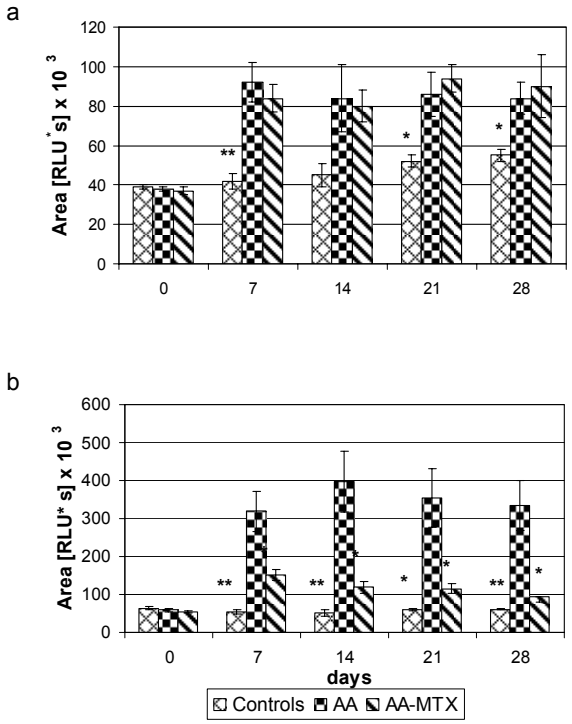

Fig. 2. **Panel a) Spontaneous whole blood chemiluminescence. Panel b) Whole blood chemiluminescence stimulated with 0.005 μmol L^{-1} PMA** (phorbol-myristate-acetate). Values given in relative luminescence units (RLU) are mean from 6 animals. Control-untreated animals, AA-rats with adjuvant arthritis, AA-MTX-rats with adjuvant arthritis treated with methotrexate. Mean ± SEM, **P*<0.05, ** *P*< 0.01 (vs AA)

fect of MTX on oxidative burst of neutrophils in animals with AA was observed on day 7 of investigation.

In monocytic cell lines, the cytotoxic antiproliferative effect of MTX was accompanied by apoptotic manifestation or enhanced ROS generation. The large difference in ROS production between T-lymphocytic and monocytic cell lines was found to be related to the extent of the apoptotic effect of MTX, giving additional support to the suggestion of higher susceptibility of T-lymphocytes to MTX treatment[5]. The suppressive effect of MTX on CL of whole blood stimulated with PMA was significantly increased from day 14 to 28 of AA. As evident from the presented results, the significant increase in CL of whole blood on day 7 precedes the clinical signs of AA, appearing on day 14 of investigation. Increase in neutrophil count correlated with CL of blood in untreated animals. MTX significantly inhibited CL in PMA-stimulated blood of AA animals, presumably due to alterations in the cellular redox state in phagocytes[3]. The antiinflammatory effect of MTX resulting in suppression of oxidative burst of blood phagocytes might be a consequence of its interaction with the cAMP-protein-kinase A-dependent adenosine inhibition of neutrophil oxidative activity via the adenosine A_{2A} receptor[6].

Despite the fact that MTX induced apoptosis of T-cell lines through oxidative stress[5], our results demonstrated that pretreatment of animals with MTX significantly depressed the oxidative burst of stimulated blood phagocytes. The inhibition of neutrophils to produce ROS correlated with the improvement in the overall health state of the animals. The possible mechanism of the protective effect of MTX on oxidative burst of neutrophils in AA will be the subject of further interest.

This study was supported by projects APVV 51-017905 and VEGA 2/7019/27.

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P40
MODULATION OF RAT BLOOD PHAGOCYTE ACTIVITY BY SEROTONIN

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Key words: phagocytes, reactive oxygen species, serotonin, serotonin receptors

Introduction

There is now incontrovertible evidence that the nervous and immune systems interact bidirectionally. One potent mediator which plays a role in regulating both the nervous and immune systems is serotonin[1]. Serotonin acts as a neurotransmitter and a neuromodulator but it appears to also have a considerable influence on constituents of the immune system. During inflammatory processes, large amounts of serotonin are released by local mast cell degranulation and aggregated platelets[2]. Serotonin modulates different aspects of both adaptive and innate immunity; however, the available data are rather controversial[3,4]. The increasing use of serotoninergic agents in therapeutics together with the accumulated evidence for a role of serotonin in the immune system emphasizes the need for immunopharmacological studies. Herein, the effect of serotonin on an oxidative burst of rat blood phagocytes was evaluated and the involvement of different serotonin receptors in the effect of serotonin on phagocytes was tested using receptors agonists and antagonists.

The effects of serotonin (serotonin creatinine sulfate salt monohydrate) in a concentration range of 10^{-7}–10^{-3} M on the CL responses of peripheral rat blood leukocytes were evaluated *in vitro*. Four stimuli of phagocyte oxidative metabolism with different mechanisms of activation – opsonized zymosan (OZP), phorbol myristate acetate (PMA), calcium ionophore A23187 (CaI) and *N*-formyl-methionyl-leucyl-phenylalanin (FMLP) were tested.

Methods

The heparinized rat blood obtained via a heart puncture was layered over the separation solution to remove erythrocytes by 1 h sedimentation at room temperature[5]. The rich plasma (buffy coat) of leukocytes was washed twice and leukocyte counts were adjusted to obtain a final concentration $1 \cdot 10^6$ ml^{-1}. The CL response of leukocytes was measured using the microtitre plate luminometer, LM-01T (Immunotech, Czech Republic) and the microtitre plate luminometr, Orion II with injector (Berthold Detection Systems GmbH, Germany) within 1.5 h after blood collection. The principle of the method is based on luminol interaction with the phagocyte-derived free radicals, which results in large measurable amounts of light. Briefly, the reaction mixture consisted of $100 \cdot 10^3$ leukocytes, 1 mM luminol (stock solution of 10 mM luminol in 0.2 M borate buffer) and one of the activators. The final concentrations of activators were selected based on our previous results: 62.5 μg ml^{-1} OZP, 9.55 μM Ca-I, 0.81 μM PMA or 2.85 μM FMLP (ref.[5]). The assays were run in duplicates. Spontaneous CL measurements in samples containing leukocytes and all other substances, but none of the activators, were included in each assay. Light emission, expressed as relative light units (RLU), was recorded continuously at 37 °C for 60 min. Each kinetic curve consisted of 100 points. The intensity of the CL reaction was expressed as the integral of the obtained kinetic curves, which corresponds to the total amount of light produced during the measurements. All data are expressed as the mean of n=6.

Results

The resulting data revealed that serotonin inhibited the CL response of rat blood phagocytes activated by OZP in a dose dependent manner (fig. 1). Similarly, serotonin inhibited the CL response of rat blood phagocytes to PMA, FMLP and Ca-I in a dose dependent manner (data not shown).

Further, the involvement of different serotonin receptors (5-HT$_1$, 5-HT$_2$, 5-HT$_3$, 5-HT$_4$, 5-HT$_5$ and 5-HT$_7$) was evaluated using various agonists and antagonists of these receptors. None of these agonists and antagonists studied exerted any direct antioxidative properties as we showed previously[6].

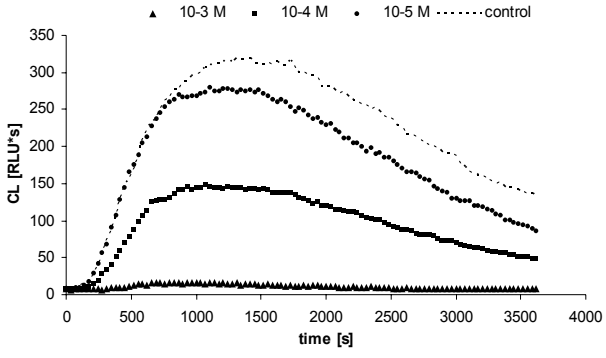

Fig. 1. **The effect of serotonin on the oxidative burst of rat blood phagocytes activated by OZP;** concentrations 10^{-6}–10^{-7} M did not differ from the control

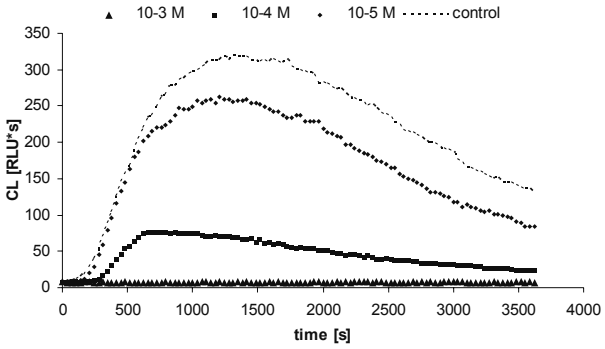

Fig. 2. **The effect of (±)-1-(2,5-dimethoxy-4-iodophenyl)-2--aminopropane hydrochloride on the oxidative burst of rat blood phagocytes activated by OZP;** concentrations 10^{-6}–10^{-7} M did not differ from the control

From all tested agonists, only the selective 5-HT_2 receptor agonist (±)-1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane hydrochloride (DOI hydrochloride) exerted a similar effect on the respiratory burst as serotonin. In concentrations of 10^{-4} M DOI hydrochloride had an even more potent inhibitory effect on CL response (fig. 2) compared to serotonin despite not having any antioxidative properties[6]. Interestingly, the application of the selective antagonist of this receptor ketanserin did not block effect of serotonin.

Conclusion

The data obtained clearly demonstrated that serotonin was a potent inhibitor of the oxidative burst of rat blood phagocytes. Since the effect of serotonin on phagocytes is complex, our experiments were focused on elucidating the possible individual mechanisms of serotonin activity. It was previously shown that serotonin could act as a true scavenger of reactive oxygen species generated during the respiratory burst of stimulated phagocytes, that it caused aggregation and degranulation of neutrophils, and it inhibited the migration of mononuclear leucocytes[7,8]. Our results suggest that the modulatory effect of serotonin on an oxidative burst of blood phagocytes occurs through the activation of the 5-HT_2 receptor subtype since DOI hydrochloride, a selective 5-HT_2 receptor agonist, had an inhibitory effect on respiratory burst similar to serotonin. However, the application of the antagonist of this receptor did not block the effect of serotonin. Therefore, we could speculate that other mechanisms are involved in the serotonin dependent modulation of rat blood phagocyte activity. Another probable explanation could be the direct scavenging activity of serotonin against free radicals produced during respiratory burst of phagocytes.

This study was executed within a research plan AVOZ50040507 and was supported by grants No. 524/04/0897 of the Grant Agency of the Czech Republic, No. 2/7019/27 of the VEGA grant agency and No. SK-CZ-06606 of the Slovak-Czech Intergovernmental cooperation.

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P41
A COMPARISON OF ANTIOXIDANT PROPERTIES OF URIC ACID, ALLANTOIN AND ALLANTOIC ACID

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Key words: uric acid, allantoin, allantoic acid, antioxidant

Introduction

Purines are the heterocyclic aromatic organic compounds, consisting of a pyrimidine ring fused to an imidazole ring. They are components of a number of important biomolecules, such as nucleic acids, adenosine triphosphate, and coenzyme A. Purines are cleared from organism by catabolic pathway, which is common to all animal species. However, during the evolutional process some of the enzymes involved in this catabolic pathway were lost which led to arresting of purine catabolism at different levels. In mammals, purine catabolism ends with allantoin, a product of uric acid degradation by the enzyme urate oxidase. The only exceptions constitute the anthropoid apes and humans in which urate oxidase activity had been completely lost. These species do not express urate oxidase due to nonsense mutations on the gene encoding it[1] and are not able to degrade uric acid to allantoin. This leads to high plasma concentrations of uric acid, which manifold exceed concentrations typical for other mammals. Although the lack of urate oxidase in apes and humans is known for many years, it remains unclear until today why these mutations have been evolutionarily accepted. It was hypothesized, that the loss of urate oxidase might constitute evolutionary advantage over other mammals because of the strong antioxidant properties of uric acid[2]. While the antioxidant properties of uric acid are well described nowadays, little is known about its catabolic products. The aim of this study was therefore to examine the antioxidant properties of allantoin and allantoic acid and to compare them with antioxidant properties of uric acid.

Materials and methods

Scavenging of peroxyl radical was measured luminometrically using the TRAP method[3]. Trolox, a water-soluble analogue of α-tocopherol was used as a standard. The data are expressed as μmol of peroxyl radicals trapped by 1 l of sample. Concentration of tested samples was 300 μM.

Scavenging of hydroxyl radical was measured luminometrically. System Fe^{2+}-EDTA + hydrogen peroxide was used to generate hydroxyl radical. The tested compound (300 μM) was mixed with luminol (1 mM), distilled water and H_2O_2 (2 mM). The reaction was started by adding of 1 mM of Fe^{2+}/1.2 mM of EDTA. The integrals of the resultant kinetic curves were used to evaluate the scavenging activity and expressed as relative light units (RLU).

Scavenging of superoxide was measured colorimetrically using XTT. Xanthine oxidase (0.04 U ml^{-1}) in PBS was mixed with EDTA (100 μM), XTT (40 μM) and sample (300 μM). The reaction was started by addition of xanthine (660 μM). The reaction mixture was incubated at lab temperature for 10 minutes and after incubation absorbance at 470 nm was measured.

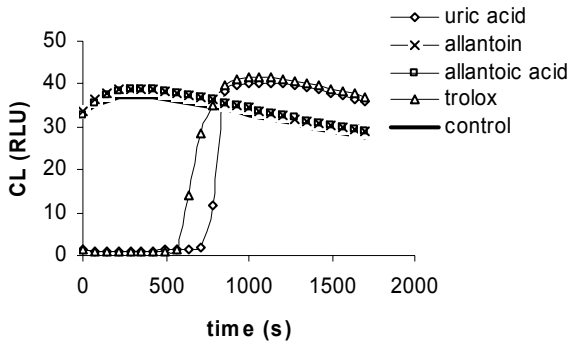

Fig. 1. **Scavenging of peroxyl radical by uric acid, allantoin and allantoic acid measured with TRAP method;** Trolox was used as reference antioxidant

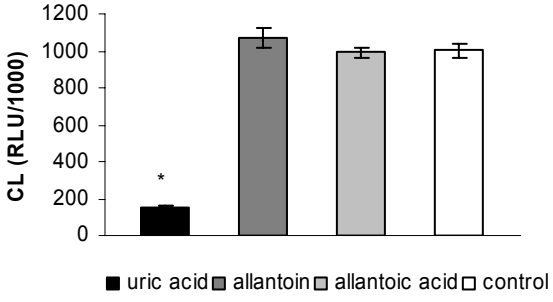

Fig. 2. **Scavenging of hydroxyl radical by uric acid, allantoin and allantoic acid measured luminometrically;** the values represent integrals of resultant kinetic curves expressed as relative light units/1000

Results

Data show that neither allantoin nor allantoic acid scavenged peroxyl radical (fig. 1). Similarly, neither allantoin nor allantoic acid scavenged hydroxyl radical, whereas uric acid was a very effective scavenger of this reactive oxygen species (fig. 2). Uric acid and allantoic acid did not react significantly with superoxide anion. Allantoin reacted significantly with superoxide when compared to control. However, when allantoin was compared to uric acid, none statistically significant difference was observed (fig. 3).

Discussion

Uric acid is known to be one of the most important antioxidants in human body fluids[4,5]. It is an effective scavenger of strong oxidants, as are hydroxyl radical, singlet oxygen[2,6], peroxyl radical, hypochlorous acid[6] and radicals derived from the reaction between peroxynitrite and carbon dioxide[7]. It was also shown to chelate transition metal ions and to inhibit iron ion-catalyzed oxidation of ascorbic acid[8]. Interestingly, at low concentrations typical for most mammals, uric acid was shown to have prooxidant activity. Prooxidant/antioxidant switch occurs approximately at concentrations between 200–400 μM (ref.[9]).

Our results imply, that neither allantoin nor allantoic acid are scavengers of hydroxyl or peroxyl radicals. Conversely, uric acid effectively scavenges these reactive oxygen species. This finding is consistent with observations of other authors[2,6]. As for superoxide scavenging, mild antioxidant action of allantoin was observed but without significant difference compared to uric acid. Skinner et al. observed that allantoin, contrary to uric acid, did not react with peroxynitrite[10]. Whiteman and Halliwell arrived at the same conclusion[11]. Overall, these results are in accordance with the hypothesis, that the arresting of purine catabolism at the level of uric acid could represent an evolutionary advantage for the species involved. However, it still needs to be considered that uric acid, allantoin and allantoic acid were compared only from the aspect of their direct antioxidant properties in our experiments. The question, which remains unanswered, is the other biological effects of tested substances independent of their antioxidant capacity.

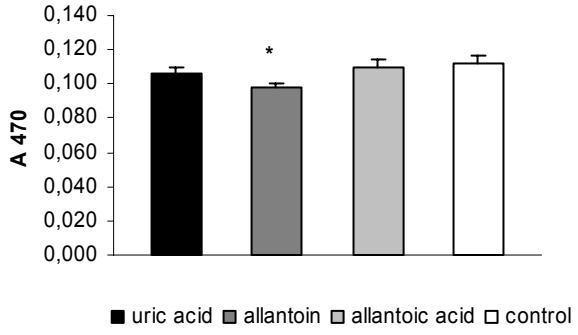

Fig. 3. **Scavenging of superoxide by uric acid, allantoin and allantoic acid measured with XTT test;** the values represent absorbances at λ = 470 nm

This study was undertaken as part of research plan AVOZ50040507 and supported by the Czech Science Foundation (grant No.204/07/P539).

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P42
DIAGNOSIS AND ELIMINATION OF THALLIUM AFTER AN INTENTIONAL INTOXICATION

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Key words: Prussian blue, excretion, neurological, alopecia

Introduction

Thallium salts very rarely cause poisoning, as their use as rodenticides has been banned in most countries. The lethal dose is about 10 mg kg^{-1} body weight, i.e. usually less than 1000 mg. Thallium is excreted in the faeces and urine, in a proportion of 2:1. The biological half-life of thallium in documented cases was in a broad range of 1–30 days[1]. Therefore, diagnosis after several months of latency is usually difficult[2–4]. There are no data on the excretion of thallium after the antidote challenge test in the "unexposed" population, where it originates from environmental sources, especially emissions from the manufacturing process of fossil fuels.

In the Czech Republic, two women recently experienced thallium intoxication[5].

First patient

44-year-old woman, developed severe pain with paresthesias in the lower limbs in November 2004. These symptoms disappeared after 3 weeks; however, in March 2005 she suddenly experienced strong muscular pain in the lower extremities, with her gait as painful "as walking on broken glass". Within 5 days she lost her hair. In August 2005, a third period of symptoms in the lower limbs developed, and she also had blurred vision. The diagnosis was not clear. After 3 weeks, mild pain and her vision difficulties still persisted.

Second patient

In December 2005, her 22-year-old daughter developed the same symptoms. Over the following 3 weeks she became unable to walk. In the 4th week she lost all of her hair, developed severely blurred vision, and could only discern fingers at a distance of 0.3 m.

Thallium was found in the urine of the younger woman. Both women suspected they had been poisoned by the father/husband, who had access to a stock of old rodenticides.

In January 2006, both women were hospitalized in our department. Treatment with the antidote Prussian blue[6], ferric hexacyanoferrate $Fe_4[Fe(CN)_6]_3$, was started in the daughter. After the first dose of 6 g, the concentration of thallium in her urine increased twofold from 580 μg l^{-1} to 1170 μg l^{-1}, i.e. to 1760 μg/12 h. Maximum thallium concentration in the faeces was 5220 μg 100^{-1} g, and the daily excretion reached 13,000 μg. Maximum total daily thallium from both urine and faeces can be seen in Table I. Antidotal treatment was continued for 22 days, until the thallium concentration in the urine dropped under 5 μg l^{-1}.

The mother was also admitted, as she was still complaining of blurred vision in the central and upper parts of the visual field and of the inability to read. Therefore, as a challenge test, the mother was given the same dose of 6 g of the antidote. Thallium in the urine, unmeasurable by voltammetry prior to treatment, increased after the antidote to 21 μg/12 h. Maximum total daily excretion in both urine and the faeces is shown in Table I.

A challenge test with 6 g of Prussian blue was performed on a control subject, a woman. After an identical dose of Prussian blue, thallium in the urine increased to only 4 μg/12 h.

By April 2006 the daughter could walk with the aid of a walker for leg support. By August 2006 she walked without support, and she could see fingers from 0.75 m.

Conclusion

Treatment with Prussian blue produced a higher excretion of thallium in both women, compared to the control subject. The challenge test with this antidote can contribute to diagnosis even after 5 months after last ingestion of thallium. The reversibility of the polyneuropathy in the lower extremities and of the vision damage is still questionable.

Table I
Measurement of thallium in biological samples of the patients and a control subject

	Units	Daughter	Mother	Normal values (ref.[1])
Days of treatment with Prussian blue		22	5	
Estimated Latency from Exposure		6 weeks	5 months	
Initial Measurement (Optical Emission Spectrometer-Inductively Coupled Plasma Analysis)				
Blood	[μg l^{-1}]	770	0.3	0.049–0.130
Urine	[μg l^{-1}]	580	8.5	0.018–0.021
Hair	[μg g^{-1}]	6.8	–	0.007–0.650
Peak With Antidote (Voltammetry)				
Urine	[μg l^{-1}]	1170	21	4*
Urine	[μg/12 h]	1750	31	4.2*
Faeces	[mg/100 g]	5.2	0.55	?
Maximum daily thallium elimination	[mg/24 h]	16.0	0.25	?
Measurement After the End of Treatment (Voltammetry)				
Urine	[μg l^{-1}]	2.0	5.4	negative
Urine	[μg/12 h]	2.4	4.0	negative
Faeces	[mg/100 g]	0.10	0.07	negative
Last measurement (Voltammetry)		August 2006	April 2006	
Urine	[μg l^{-1}]	negative	negative	negative
Urine	[μg/12 h]	negative	negative	negative

– not measured, ? not known, *measurement in control subject

Supported by grant MSM 0001620807.

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P43

PROGRESS OF PROCESS TO DETERMINATION OF INHALATION ACRYLATE EXPOSURE AS BASEMENT METHOD FOR HUMAN RISK ASSESSMENT

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Introduction

Workers in dental material production, technicians in stomatological laboratories and surgeries are exposed to monomer of methyl methacrylate (MMA)[1,2]. This monomer is the main component of stuffing resins, toothprint material and acrylate teeth. Monomer usage in stomatology is very different from industrial application. There are higher concentration of MMA under laboratory conditions. There is also contact of MMA with worker's skin. MMA is clear, colourless liquid with a acrid odour, extremely volatile organic compound irritated skin, mucosa and eyes, caused chronic conjuctivitis[3,4]. During exposition there is happened skin intrusion[5]. In present there is no human epidemiological study for MMA. Completed workplace studies inform us of appearance of worker's dermatological problems[1,2]. Nobody occupied with concentration monitoring and MMA exposition on workplace. Vast majority of publication is concerned in toxicity tests on animals[6–8].

Measurement of workplace with methyl methacrylate exposure

In first stage of study there were followed out measurements of MMA in company produces acrylic teeth. Monitored workplaces were "matter preparation", "pre-bundle press" and "teeth press". There is three-shift production with working time 7.5 hours. Workers are exposed to MMA through the whole shift. There are totally exposed seventy workers in these workplaces. In room "matter preparation" the monomer is mixed with polymer (resin powder – polymethylmethacrylate) in stirring machine. Monomer is liquid mixture of MMA and 1,4-buthandioldimethylacrylate (BDMA) in mass representation 91 % MMA and 9 % BDMA. Matter is filled into the polyethlyene tube and then kept in cool in refrigerator. In "pre-bundle press" the matter is treated to shape for final teeth press in special devices. Next step is control polymerization of resin matter in presses.

Sample collection was carried out by personal sampling in worker´s expiratory zone. Expiratory zone is semispheric area with average 30 centimeter[8]. In this study there were used SKC pumps type 224-PCXR. These pumps are able to work with constant air flow. Sorptive material was granular activated carbon.

Methyl methacrylate determination in air by gas chromatography method

Methyl methacrylate captured on activated carbon was desorbed by extraction to solvent (CS_2). Its content is determinated by GC-MS method. Gas chromatograph GC-210 with mass detector GCMS-QP 2010S with column DB5-ms (J&W Scientific) was used.

Calibration standard was methyl methacrylate with <99 % purity. Accurate concentration is found by differential weighing to standard flask with carbon sulfide. Calibration standard is injected directly on gas chromatograph column (injection split – 1 µl, injection temperature 250 °C).

Results were calculated from obtained chromatograms by external standard method or by method of standard addition with respected sample dilution by evaluation station "GCMS solution". Retention times are evaluated and peak areas of analyzed compounds are compared. Results are reported in mass concentration mg/sample. Mass concentration mg m^{-3} is calculated from sucked air data.

Measured and limiting concentration comparison

Summary of maximal and all-shifted MMA concentration from chosen workplaces are written in the Table I. Periodic measurements were carried out on workplaces where limiting concentrations were exceeded and always after technical actions contributing to decrease MMA concentration on workplace. Hygienic limits in Czech legislation for MMA on workplace air are: the highest permissible concentration NPK-P 150 mg m^{-3}, permissible exposure limit PEL 50 mg m^{-3}. According table number one the value PEL is still exceeded on workplace "matter preparation". The value is excceded over 88 %.

Discussion

Thanks to the results from measurements and realized treatments on workplaces (increasing of ventilator capacity, fume cupboard installation above the work table), the concentration of MMA were decreased. In spite o fit the MMA values on workplace "matter preparation" of all-shift concentration are still over the limit. One reason is frequent manipulation with liquid monomer MMA in opened vessels outside of fume cupboard. MMA is extremely volatile compound. This workplace in enclosed – without windows. Change of air is happened by ventilators. On the other side their power is limited by microcli-

Table I
Summary of maximal measured concentration and all-shift concentration

Workplace	Maximal measured concentration c_{max} [mg m^{-3}]	All-shift concentration c [mg m^{-3}]
Matter preparation	238.636	224.56
	141.026	107.885
	100.694	94.753
Pre-bundle press	167.119	157.259
	11.407	10.734
Teeth press	30.820	29.0

matic requirements on workplace (circulation, temperature). It is impossible to increase their capacity. It can not be ruled out that MMA vapors diffused through the ventilators to other workplaces. MMA is still released to external environment before final press. Whichever manipulation with this matter brings risk of MMA exposition. These facts obliged company with these productions to discuss what to do. First step will be very large monitoring of MMA concentration. Measurement will be done step by step through the whole production. Before monitoring evolution of device capable of passive sampling of followed compound will be done. This device uses effect of molecular diffusion and is called passive dosimeter. Followed compound diffused to the surface of sorption material is placed to the diffuser according to the gradient of concentration. Passive dosimeter is small and light device which is ease to fix on worker's dress. Usage with it is very simple. In this study there will be also completed biological monitoring of MMA. Biological monitoring will be done by cytogenetical analysis of peripheral lymphocytes. This analysis is used for biological monitoring of genotoxical factors in municipal place and workplace. Presence of genotoxical active compound in place affected human body is probed by frequency of chromosomal aberration in monitoring group.

Conclusion

This article has been trying to introduce basement of problem happened on workplace with liquid MMA manipulation under laboratory conditions. It is not only teeth production but also work of laboratory technicians and dentists. Production is happened under laboratory conditions but MMA amount using there is very dangerous because of its volatility. Epidemiological studies were done mainly on animals. Sampling in workplace with personal pumps is very expensive. Number of parallel samples is limited by number of pumps. Workplace monitoring is suitable to realize in one period under the same conditions (work, climatic and microclimatic conditions, ...). Because of penetration of MMA through the skin, it is suitable not to realize only sampling in air but to do biological monitoring of compound in human organism.

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P44

COMPARISON OF THE CYTOTOXICITY OF AND DNA ADDUCT FORMATION BY THE ANTICANCER DRUG ELLIPTICINE IN HUMAN BREAST ADENOCARCINOMA, LEUKEMIA AND NEUROBLASTOMA CELLS

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Key words: ellipticine, MCF-7, HL-60, CCRF-CEM and neuroblastoma cells, peroxidases, cytochromes P450, DNA adduct

Introduction

Ellipticine (5,11-dimethyl-6*H*-pyrido[4,3-*b*]carbazole, fig. 1), an alkaloid isolated from *Apocyanaceae* plants, exhibits significant antitumor and anti-HIV activities[1,2]. The main reason for the interest in ellipticine and its derivatives for clinical purposes is their high efficiencies against several types of cancer, their rather limited toxic side effects, and their complete lack of hematological toxicity[1]. Nevertheless, ellipticine is a potent mutagen (for review see[1,2]). Ellipticines are anticancer drugs, whose precise mechanisms of action have not been explained yet. It was suggested that the prevalent mechanisms of their antitumor, mutagenic and cytotoxic activities are (*i*) intercalation into DNA and (*ii*) inhibition of DNA topoisomerase II activity (for review see[1]). Ellipticine and its metabolite 9-hydroxyellipticine also cause selective inhibition of p53 protein phosphorylation in several human cancer cell lines and this correlates with their cytotoxic activity. Ellipticines also uncouple mitochondrial oxidative phosphorylation and thereby disrupt the energy balance of cells (for review see[2]).

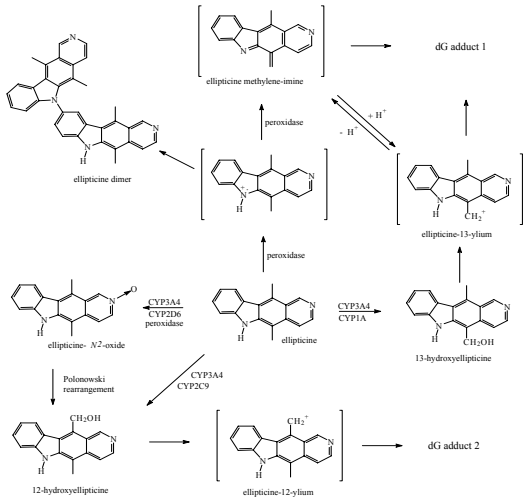

Fig. 1. **Metabolism of ellipticine by human CYPs and peroxidases showing the characterized metabolites found to form DNA adducts**

We demonstrated that ellipticine covalently binds to DNA after being enzymatically activated with cytochrome P450 (CYP) enzymes or peroxidases[2–6]. Human and rat CYPs of 1A and 3A subfamilies are the predominant enzymes catalyzing oxidation of ellipticine either to metabolites that are excreted from organisms (7-hydroxy- and 9-hydroxyellipticine) or form DNA adducts (12-hydroxy- and 13-hydroxyellipticine)[2–5]. Of the peroxidases, human cyclooxygenase (COX)-2, ovine COX-1, bovine lactoperoxidase, human myeloperoxidase and horseradish peroxidase efficiently generated ellipticine-derived DNA adducts (fig. 1)[6]. The same DNA adducts by ellipticine were also detected in V79 Chinese hamster lung fibroblasts transfected with human CYP3A4, 1A1 and 1A2 (ref.[7]) and *in vivo* in rats exposed to this anticancer drug[4,8]. Our recent studies also indicate that ellipticine is toxic to human breast adenocarcinoma MCF-7 cells[9] and leukemia HL-60 and CCRF-CEM cells[10]. On the basis of these data, ellipticine might be considered a drug, whose pharmacological efficiency and/or genotoxic side effects are dependent on its enzymatic activation in target tissues.

Here, we investigated the potential of ellipticine to damage another type of cancer cells, human neuroblastoma cells, and examined whether DNA adducts are formed in these cells. In addition, we compare toxic sensitivity of these and MCF-7, HL-60 and CCRF-CEM cells to ellipticine.

Materials and methods

Commercial MCF-7, HL-60 and neuroblastoma IMR-32 cells and the CCRF-CEM, a T lymphoblastoid cell line (from the Department of Pediatric Hematology and Oncology), cultivated in the presence of 0–10 μM ellipticine and tumor cell viability were evaluated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazoliumbromide test as described[9,10]. DNA from cells was isolated by the phenol/chloroform extraction method[2]. ^{32}P-postlabeling analyses of ellipticine-derived DNA adducts were performed using nuclease P1 enrichment as described previously[2–10]. Immunoquantitation of CYPs (CYP1A1, 1A2, 2B, 2E1 and 3A), NADPH:CYP reductase and COX-1 and -2 in homoge-

nates of cancer cells was done by Western blot as described[9,10]. Myeloproxidase (MPO) was detected by flow cytometry using anti-human MPO-FITC antibody[10].

Results and discussion

Toxicity of ellipticine to all analyzed cell lines was dose-dependent; ellipticine is the most toxic to neuroblastoma IMR-32 cells [a parent neuroblastoma cell line as well as its daughter line resistant to doxorubicine, IMR-32 (DOXO)], followed by leukemia HL-60 cells, breast adenocarcinoma MCF-7 cells and leukemia CCRF-CEM cells.

Table I
DNA adduct formation by ellipticine in human cancer cell lines and its cytotoxicity to these cell lines

Cells	IC_{50} [μM]	Total DNA adduct levels [relative adduct labeling x 10^{-7}]
IMR-32	0.26	13.1
IMR-32 (DOXO)	0.53	10.0
HL-60	0.64	64.6
MCF-7	1.25	9.3
CCRF-CEM	4.27	7.3

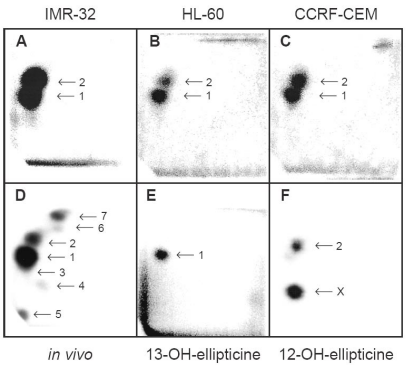

Fig. 2. **Autoradiographs of PEI-cellulose TLC maps of ^{32}P-labeled digests of DNA** isolated from neuroblastoma IMR-32 cells exposed to 10 μM ellipticine (A), HL-60 cells exposed to 1 μM ellipticine (B), CCRF-CEM cells exposed to 5 μM ellipticine (C) for 48 h, of liver DNA of rats treated with 40 mg ellipticine per kilogram body weight (D), from calf thymus DNA reacted with 13-hydroxyellipticine (E) and 12-hydroxyellipticine (F). Analyses were performed by the nuclease P1 version of the ^{32}P-postlabeling assay. (A,B,D) Scans of the plates for 6.5 min from the imager; (C,E,F) autoradiographs of films exposed for 1 h at -80 °C. Origins are located at the bottom left corners (D3 from bottom to top and D4 from left to right)

The IC_{50} value for ellipticine was up to one order of magnitude lower in IMR-32 and HL-60 cells than in MCF-7 and CCRF-CEM cells (Table I).

Using the nuclease P1 version of the ^{32}P-postlabeling assay we found that the ellipticine-derived DNA adducts were generated in all cell lines analyzed in the study (see figure 2A, B and C for IMR-32, HL-60 and CCRF-CEM cells, respectively). This assay yielded a pattern of ellipticine-DNA adducts with two major adducts, similar to the pattern of adducts detected in DNA reacted with ellipticine activated with CYP enzymes or peroxidases *in vitro*[2,5,6] and in DNA *in vivo*, in rats treated with ellipticine (fig. 2D). The two adducts formed in DNA of analyzed cells are identical with those formed by the ellipticine metabolites 13-hydroxy- and 12-hydroxyellipticine with deoxyguanosine in DNA *in vitro* (fig. 2e and f) as confirmed by HPLC of the isolated adducts (not shown). The highest total levels of DNA adducts were generated by ellipticine in HL-60 cells, followed by those of IMR-32, MCF-7 and CCRF-CEM cells (Table I).

Because the two major adducts formed from ellipticine in all tested cell types are identical to adducts derived from 13-hydroxyellipticine or 12-hydroxyellipticine, metabolites of ellipticine formed by CYP enzymes of 3A and 1A subfamilies[2–5] or from metabolites generated by peroxidases such as MPO and COX-1 and -2 (ref.[6]) (fig. 1), we analyzed the expression of these enzymes in studied cell lines. Each of the tested cancer cell lines contains at least one of the enzymes activating ellipticine. Expression of MPO protein in HL-60 cells was proven by flow cytometry using an anti-human MPO-FITC antibody (not shown). HL-60 cells also contain another peroxidase, COX-1, the expression of which was proven by immunoquantitation using an anti-COX-1 antibody (fig. 3). In contrast to this peroxidase, Western blots with polyclonal antibodies raised against COX-2 and various CYPs (CYP1A1, 2B4, 2E1 and 3A4) showed that CYP1A1 only is expressed in HL-60 cells (fig. 3).

No detectable expression of MPO was found in CCRF-CEM cells by FACS analysis. However, the Western blot analyses of other peroxidases (COX-1 and -2) and of CYP (CYP1A1, 2B4, 2E1 and 3A4) enzymes in CCRF-CEM cells revealed that COX-1 and low but detectable

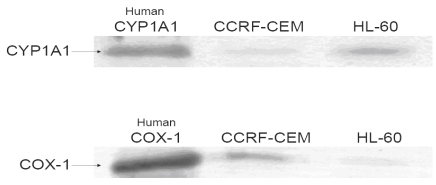

Fig. 3. **Immunoblots of CYP1A1 and COX-1 in HL-60 and CCRF-CEM reacted with antibodies against CYP1A1 and COX-1.** Cell homogenates were subjected to SDS-PAGE, proteins transferred to PVDF membranes and probed with antibodies as described in Material and Methods. Human CYP1A1 and human COX-1 were used as standards

levels of CYP1A1 are expressed in these cells (fig. 3). MCF-7 and neuroblastoma cells express only CYP1A1 (data not shown).

Conclusion

The results presented here demonstrate the formation of covalent DNA adducts with ellipticine in human cancer cell lines (breast adenocarcinoma MCF-7 cells, leukemia HL-60 and CCRF-CEM cells and neuroblastoma IMR-32 cells), and suggest the formation of covalent DNA adducts as a new mode of antitumor action of ellipticine for cancer.

Supported by the GACR (grants 203/06/0329) and the Czech Ministry of Education (grants MSM0021620813, MSM0021620808 and 1M4635608802 - Centrum of targeted therapeutics).

Abbreviations

CYP	cytochrome P450
MPO	myeloperoxidase
COX	cyclooxygenase
IC_{50}	inhibitor concentration eliciting 50 % inhibition
FITC	fluorescein isothiocyanate
FACS	fluorescence-activated cell sorting

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MITOCHONDRIAL BIOENERGETICS OF SKELETAL MUSCLE STUDIED IN ADJUVANT ARTHRITIS

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Key words: adjuvant arthritis, mitochondrial bioenergetics, oxidative stress, stimulated mitochondrial respiration, oxidative phosphorylation rate

Introduction

Rheumatoid arthritis (RA) is a chronic relapsing immuno-inflammatory multi-system disease with predominant synovial proliferation and destruction of articular cartilage[1]. The exact etiology of RA remains unknown. In chronic persistent synovial inflammation that either a foreign agent or some alteration in control of cellular responses, possibly genetically mediated, has been implicated[2]. Oxidative stress and redox imbalance are considered to contribute to the pathogenesis of chronic inflammatory diseases including RA. Numerous studies have suggested a role for oxidant stress in the pathogenesis of RA (ref.[3,4]). Several reviews have discussed the importance of oxidative stress and redox signaling in vascular inflammation and, in general, have suggested the involvement of NADPH oxidase, xanthine oxidase, mitochondrial respiration, cyclooxygenases and lipoxygenases in the production of vascular reactive oxygen species[5–7]. In general, inflammatory mediators present in inflamed synovial tissue, such as pro-inflammatory cytokines (i.e. TNF-α) and growth factors, activate the intracellular production of reactive oxygen and nitrogen species[8].

Oxidative stress present in rheumatoid arthritis also participates in changes of mitochondrial bioenergetics. These changes lead to a dramatic reduction of physical performance in patients with RA, due to atrophy of skeletal muscles[9].

Aim

In this study we compare the data obtained in different measurements of mitochondrial bioenergetics in skeletal muscle. We selected three independent experiments with different levels of injury in the model of adjuvant arthritis (AA). Clinical parameters (decrease of body weight and hind paw volume) and biochemical parameters, i.e. stimulated mitochondrial respiration (state 3) and the rate of ATP production (OPR − oxidative phosphorylation rate) for complex I (NAD-glutamate) and complex II (FAD-succinate) in mitochondrial respiratory chain, were monitored and compared.

Materials and methods

AA was induced in male Lewis rats (Breeding Farm Dobrá Voda, Slovakia), weighing 150–170 g, by a single intradermal injection of heat-killed *Mycobacterium butyricum* in incomplete Freund´s adjuvant. The three independent experiments[9–12] included healthy animals as reference controls (C) and arthritic animals without any drug administration (AA). We monitored clinical parameters, i.e. change of body weight (CBW) and hind paw volume (HPV). CBW was calculated as the difference of body weight measured on day 28 and body weight measured at the beginning of the experiment. The HPV increase was calculated as the percentage increase of HPV on day 28 in comparison to that at the beginning of the experiment. Mitochondria of the skeletal muscle were isolated by differential centrifugation. Respiratory chain function was measured using Clark oxygen electrode[13]. The data for all parameters were expressed as arithmetic mean and SEM. For significance calculations, Student`s t-test was used with $*P<0.05$ (significant); $**P<0.01$ (very significant); $***P<0.001$ (extremely significant). The arthritic groups were compared to healthy control animals. All parameters were compared as ratios of arthritis to control, excluding the CBW. For this parameter the difference between the control and arthritic animals was used.

Results and dscussion

We selected three independent experiments[9–12] with different levels of injury in the model of adjuvant arthritis. The clinical data are given in Table I. Ratios for HPV and differences of CBW were summarized, and on the basis of their values we classified the three levels of adjuvant arthritis as mild (AA_1)[9], medium (AA_2)[10,11] and severe injury (AA_3)[12]. Stimulated mitochondrial respiration (state 3) and the rate of ATP production (OPR) for complex I (NAD) and complex II (FAD) in mitochondrial respiratory chain are given in Table II and Table III. Mild injury in AA resulted in increase of functional parameters of mitochondrial bioenergetics, whereby the differences between healthy animals and animals with AA were very significant. We explain this finding as a consequence of adaptative mechanisms against oxidative stress caused by AA. Medium injury in AA induced increase in stimulated mitochondrial respiration for complex I. In the other biochemical parameters, only not significant differences were recorded between healthy and arthritic animals. In severe

Table I
Comparison of the severity of adjuvant arthritis using clinical parameters (HPV and CBW)

Groups of animals	HPV [%]	CBW [g]
C_1	22.67 ± 1.71	69.5 ± 2.63
AA_1	65.69 ± 8.63***	37.75 ± 8.76***
AA/C resp. C-AA	2.9	31.75
C_2	14.13 ± 2.11	91.43 ± 4.35
AA_2	56.79 ± 12.92**	31.0 ± 13.93**
AA/C resp. C-AA	4.02	60.43
C_3	17.20 ± 1.81	61.33 ± 10.26
AA_3	80.94 ± 8.7***	-5.11 ± 3.74***
AA/C resp. C-AA	4.71	66.44

HPV – hind paw volume, CBW – change of body weight. Values are mean ± SEM; significance AA compared to control: *P≤0.05, **P≤0.01, ***P≤0.001

Table II
Changes in skeletal muscle mitochondrial function (state 3 and OPR) for complex I (NAD-glutamate) induced by adjuvant arthritis

Groups of animals	State 3 [nAtO/mg prot./min]	OPR [nmol ATP/ mg prot./min]
C_1	92.6 ± 6.16	215.9 ± 19.2
AA_1	151.1 ± 13.4**	354.8 ± 25.9**
AA/C	1.63	1.64
C_2	46.6 ± 9.5	130.0 ± 20.7
AA_2	72.9 ± 11.3	152.0 ± 26.8
AA/C	1.56	1.17
C_3	64.8 ± 4.85	218.8 ± 16.6
AA_3	51.6 ± 4.22	174.3 ± 17.1
AA/C	0.80	0.80

State 3 – stimulated mitochondrial respiration, OPR – oxidative phosphorylation rate. Values are mean ± SEM; significance AA compared to control: *P≤0.05, **P≤0.01, ***P≤0.001

injury of arthritic animals, accompanied by 5-times higher hind paw volumes and decreasing body weight compared with healthy animals, mitochondrial functions tended to be inhibited. This may be explained by exhaustion of adaptative mechanisms of mitochondrial bioenergetics.

Conclusion

Our results indicate a dependence of functional capacity of mitochondria on the severity of induced AA.

This work was supported by grants: VEGA 2/5051/25, VEGA 1/3442/06, APVV-51-017905.

Table III
Changes in skeletal muscle mitochondrial function (state 3 and OPR) for complex II (FAD-succinate) induced by adjuvant arthritis

Groups of animals	State 3 [nAtO/mg prot./min]	OPR [nmol ATP/ mg prot./min]
C_1	111.1 ± 3.81	130.4 ± 7.04
AA_1	186.7 ± 17.2**	245.2 ± 22.2**
AA/C	1.68	1.88
C_2	134.5 ± 22.7	173.6 ± 25.4
AA_2	127.4 ± 17.6	191.2 ± 27.4
AA/C	0.95	1.1
C_3	85.05 ± 8.53	167.9 ± 17.9
AA_3	77.7 ± 4.45	168.5 ± 13.1
AA/C	0.91	1.0

State 3 – stimulated mitochondrial respiration, OPR – oxidative phosphorylation rapidity. Values are mean ± SEM; significance AA compared to control: *P≤0.05, **P≤0.01, ***P≤0.001

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