Effects of hypoxia and metabolic (or mitochondrial) inhibitors on ATP content of renal medulla in isolated perfused rat kidneys. ATP levels were measured in separate experiments, using kidneys perfused for 20 min, rapidly frozen by immersion in liquid nitrogen, medulla separated from cortex by free hand dissection while frozen, and assayed by an enzymatic method.[12] These short perfusions were designed to obtain ATP levels as an early parameter of metabolic inhibition (presumably less affected by loss of cellular ATP resulting from later secondary injury). In additional determinations at 90 min of perfusion (not shown), rotenone or 2-deoxyglucose compared to controls produced ATP depletion similar to that observed at 20 min of perfusion. While the level of ATP in frozen kidney tissue may not correspond precisely to the ATP content of cells of the medullary thick ascending limb, rotenone and antimycin have been shown to deplete ATP in isolated proximal tubules[8] and to stop respiration in isolated in TAL cells from rabbit kidney (Lear, S. and Silva, P., unpublished observations). The results are expressed as means ± SE and were analyzed by a multiple comparison procedure (Walker-Duncan)

	Medullary ATP (μM/100 mg protein)	Number of kidneys perfused
Control perfusions	1.48 ± 0.08	9
Hypoxic perfusions	0.69 ± 0.03*	6
Cyanide (2.5 mM)	0.76 ± 0.05*	4
Rotenone (10^{-5} M)	0.72 ± 0.06*	6
Antimycin (10^{-5} M)	0.70 ± 0.04*	4
Oligomycin (10^{-4} M)	0.88 ± 0.07*	5
2-deoxyglucose (50 mM)	0.88 ± 0.05*	4
Malonate (25 mM)	1.09 ± 0.07*	5
Monofluoroacetate (5 mM)	0.98 ± 0.07*	6
Antimycin and 2-deoxyclucose[a, b]	0.50 ± 0.02*†	5
Rotenone and 2-deoxyglucose[a, b]	0.48 ± 0.01*†	5

[a]Glucose was excluded from the perfusion medium; [b]Same concentrations as in previous experiments; *Significantly lower ($p < 0.05$) than control perfusions; †Significantly lower ($p < 0.05$) than hypoxic perfusions, rotenone and antimycin.

oxic damage[3] may not simply relate to a more rapid exhaustion of cellular energy stores but possibly to further stimulation of mitochondrial activity. This mode of injury appears to depend on continued mitochondrial electron flow in the face of limited oxygen supply, a situation which may conceivably lead to aberrant energy biotransformation such as the production of electron-dependent free radicals. Metabolic arrest, by hypothermia or inhibition of cell activity, which confers protection from ischemia in various tissues[1,2,7,15], may be mediated at least in part by a reduction in hypoxic mitochondrial respiration.

While it is clear that anoxia can damage cells by depletion of energy stores[4,14], some cells such as the mTAL and cultured hepatocytes[16] withstand mitochondrial inhibition far better than they do anoxia. Mitochondrial activity in the face of oxygen deprivation may be in itself an important determinant of anoxic injury.

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The protective effect of Thiola against the genotoxic action of benzo(a)pyrene

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Summary. The protective effect of Thiola against the genotoxicity, induced by benzo(a)pyrene, in vitro and in vivo, was investigated. By association of Thiola to benzo(a)pyrene a significant decrease of the numerical and structural chromosome aberrations and a reduction of the incidence of c-mitoses has been obtained in human diploid cells, i.e. human embryonic lung fibroblasts of the cell-line ICP-23, and C_{56}Bl/6 mouse bone marrow cells.

Key words. Chemoprophylaxis; cancer prevention; benzo(a)pyrene; chromosomal aberrations; genotoxicity; Thiola; anticarcinogens.

It is well known that benzo(a)pyrene (B(a)P), a widespread environmental precarcinogen, is converted in animal and human tissues by the P-450 dependent monooxigenase system[1] into electrophilic species which represent the active genotoxic metabolites[2,3]. The activation takes place simultaneously with the detoxication which transforms some B(a)P intermediates into hydrophilic derivatives which are subsequently excreted as conjugates[4].

The implication of SH-physiological compounds (i.e. glutathione, cysteine, etc.) in the detoxication processes was demonstrated and widely accepted[5,6].

The aim of the present study was to demonstrate the protective effect of a synthetic thio-compound: Thiola (N-2-mercaptopropionyl glycine) against the genotoxic action produced by B(a)P in vitro and in vivo systems.

Materials and methods. Cell culture: human diploid cells (HDC) i.e. human embryonic lung fibroblasts of the cell line ICP-23[7,8], at their 19th and 20th passages were cultivated as a monolayer in

Table 1. The genotoxic action of B(a)P and B(a)P + Th in diploid human embryonic lung fibroblasts (IPC-23)

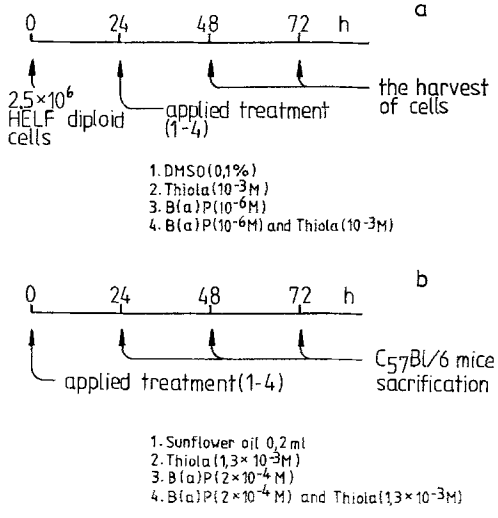
Treatment	Time of action (h)	Cells with multiple aberrations (%)	Hyperploid cells (%)	Cells with c-mitosis (%)	Structural chromosome aberrations per cell					
					Chromatid-type			Chromosome-type		
					Gap	Break	Exchange	Gap	Break	Exchange
DMSO	24	0	0	0	0.02	0.06	0.04	0.02	0.10	0.66
	48	0	0	0	0	0.02	0.08	0	0.02	0.24
Th	24	0	0	0	0.04	0.06	0.06	0	0	0.66
	48	0	0	0	0	0.10	0.90	0.07	0.06	0.44
B(a)P	24	26	2	4	0.08	0.27	2.67	0	1.19	4.59
	48	32	4	2	0.26	0.82	2.08	0.03	0.20	3.09
B(a)P + Th	24	8	2	0	0.11	0.37	1.80	0.04	0.04	1.37
	48	14	0	0	0.11	0.23	1.25	0	0.04	1.37

Eagle's minimal essential medium supplemented with 10% calf serum (v/v), 100 U/ml of penicilin and 100 µg/ml of streptomycin. All cultures were mycoplasma-free.

Confluent cultures were trypsinized (0.25% trypsin solution, 1:250 Gibco), reseeded at a density of 2.5×10^6 cells into Powitzky 50 cm^2 flasks in 25 ml medium (3 flasks per experimental point) and treated 24 h later as follows: a) B(a)P (Merck, Darmstadt, West Germany) at a final concentration of 10^{-6}M; b) B(a)P (10^{-6}M) and Thiola (Th) (Buchs, Switzerland) dissolved in medium, filter sterilized and diluted to a final concentration of 10^{-3}M; c) Th(10^{-3}M); d) DMSO (0.25 ml), as shown in figure 1a. After treatment, the cells were further incubated for 24 h and 48 h at 37 °C.

Before the cells were harvested all cultures were incubated for 2–3 h in the presence of 0.25 µg/ml colchicine (Buchs, Switzerland). The cells were submitted to trypsinitation followed by a 12 minute exposure to a hypotonic solution of distilled water ÷ medium (2:1), then fixed with methanol:glacial acetic acid (3:1). Chromosome slides were prepared according to the routine method[9] and stained with 10% Giemsa (Merck, Darmstadt, West Germany) solution.

Animal treatments. Mice (C_{57}Bl/6, male) 7–10 weeks old with 20 ± 2 g b.wt. were randomly divided in to four groups which received (fasting) the following treatments: a) 2×10^{-4}M/mouse B(a)P (dissolved in sunflower oil) administered orally; b) 1.3×10^{-3}M Th (dissolved in saline isotonic solution) injected i.p. after B(a)P (2×10^{-4}M) administration; c) Th(1.3×10^{-3}M) alone and d) sunflower oil (0.2 ml/mouse) (fig. 1b).



a) Treatment of human embryonic lung fibroblast (ICP-23 cell line). b) Treatment of C_{57}Bl/6 mice.

The mice were killed 24, 48 and 72 h after the treatment. Mitotic arrest was achieved 2–3 h before killing by i.p. injection of 2 mg/kg b.wt of colchicine. The mouse femoral bone marrow cells were collected, washed with PBS at 37 °C, exposed 12 min to hypotonic solution (KCl 0.075 M) fixed in four changes of methanol: glacial acetic acid (3:1), spread using the method described by Hungerford[10] and stained with 10% Giemsa.

In order to determine the incidence of structural chromosome aberrations, a minimum of 50 metaphases were examined for each experimental point both in vitro and in vivo. The structural chromosome aberrations were classified into chromatid and chromosome gaps (achromatic lesions) and breaks (displacement of a chromatid fragment, or when the distance between two parts of a chromatid arm was larger than the diameter of the chromatid). Asynchronous centromere separations were counted as chromosome breaks. Chromatid exchanges included: asymmetrical chromatid exchange, triradial, symmetrical and asymmetrical quadriradial. Chromosome exchanges were considered: dicentric and ring chromosomes, minute chromosomes or double minute chromosomes. Metaphases containing anaphasic chromosomes were counted as c-mitoses and cells with more than ten structural chromosome aberrations as cells with multiple aberrations.

The statistical significance of the difference between the incidence of structural chromosome aberrations in protected and nonprotected HDC and mouse bone marrow cells was calculated with the chi-square test[11].

Results. The frequency of chromosome aberrations obtained in vitro in all experiments is given in table 1. After 24 h B(a)P treatment a marked incidence of aberrant metaphases occurred (mostly dicentric and ring chromosomes, as well as chromosome breaks) and 26% of all scored cells were found to have multiple aberrations.

In 48 h B(a)P exposed HDC the chromatid-type aberrations (mostly breaks and gaps) were increased. The number of cells with multiple aberrations was 32% and 4% hyperploid cells were scored. The incidence of c-mitosis decreased from 4% (at 24 h) to 2% (at 48 h).

The association of Th with B(a)P treatment significantly decreased the clastogenic effect of B(a)P at 24 h ($p < 0.001$) and at 48 h ($p < 0.001$). No hyperploid cells (at 48 h) or c-mitosis (at 24 and 48 h) were observed (table 1).

Th, as well as other thio-compounds, induced by itself some structural chromosome aberrations[12].

The analysis of chromosome abnormalities induced by B(a)P in mouse bone marrow cells (table 2) showed a marked increase in chromosome-type aberrations (especially chromosome exchanges and breaks) at 24 and 72 h after treatment. A decreased incidence of chromosome aberrations, except chromatid exchanges, was obtained at 48 h, when only 2% metaphases have multiple aberrations. The most c-mitoses (12%) were scored at 48 h after B(a)P administration.

The addition of Th to B(a)P treatment reduced the incidence of

Table 2. The genotoxic action of B(a)P and B(a)P + Th in $C_{57}Bl/6$ mouse bone marrow cells

Treatment	Time of action (h)	Cells with multiple aberrations (%)	Hyperploid cells (%)	Cells with c-mitosis (%)	Structural chromosome aberrations per cell					
					Chromatid-type			Chromosome-type		
					Gap	Break	Exchange	Gap	Break	Exchange
Oil	24	0	0	0	0	0.02	0.04	0	0.02	0.02
	48	0	0	0	0	0.02	0.10	0	0.02	0.12
	72	0	0	0	0	0	0.12	0	0.02	0.12
Th	24	0	0	0	0.17	0.26	0.08	0.10	0	0.86
	48	0	0	0	0.04	0.18	0	0	0.06	0.16
	72	0	0	0	0.08	0.38	0.08	0.10	0.06	0.30
B(a)P	24	14	4	4	0.42	0.81	1.42	0.23	0.30	2.21
	48	2	2	12	0.02	0.49	1.51	0	0.12	0.73
	72	4	2	2	0.25	0.85	1.25	0.14	0.29	1.37
B(a)P + Th	24	0	2	4	0.06	0.84	0.64	0.08	0.06	0.96
	48	0	2	2	0.06	0.28	0.30	0.02	0.04	0.34
	72	0	2	2	0	0.26	0.64	0.06	0.10	0.52

chromosome abnormalities, except the chromatid breaks at 24 h and chromatid exchanges at 72 h. The number of c-mitoses decreased at 48 and 72 h (2%). No cells with multiple aberrations were observed after treatment with B(a)P-treatment together with Th (table 2).

The X^2 test revealed significant differences ($p < 0.001$ at 24 and 72 h and $p < 0.05$ at 48 h) in the frequency of structural chromosome aberrations between the mice treated with B(a)P and those treated with B(a)P + Th.

Discussion. Previous results demonstrate that the metabolites of B(a)P produce chromosome abnormalities[13–16].

Our results demonstrate that B(a)P induces chromosome aberrations and c-mitosis both in vitro and in vivo, and the protective effect of Th against the clastogenic and c-mitotic action of B(a)P in HDC and in bone marrow cells of mice.

The data presented show that at 24 h in vitro and in vivo B(a)P induced mainly chromosome-type aberrations. Because in all experiments asynchronously growing cell populations were used, we suppose that the aberrations occurred during the G_1 phase of the cell cycle and it is possible that B(a)P slows down the progress of cells though the S phase[17]. Our results are in agreement with those of Gehly[18], who found a high incidence of chromosome aberrations in first mitoses after B(a)P treatment and a lower one in second mitoses. This fact suggests either cell death, or that DNA synthesis and cell division were drastically reduced after the formation of adducts. Rüdiger[19] and Tompa[20] observed at 48 h after B(a)P administration an increase of detoxication by the formation of hydrophilic metabolites. In contrast, the increase of the incidence of chromosome abnormalities at 72 h could be explained by B(a)P and/or its active metabolites recycling in mouse bone marrow cells, or by a decrease of the detoxication efficiency.

The c-mitosis activity of B(a)P found in vitro and in vivo suggests the binding of the electrophilic B(a)P metabolites to tubulin (one of the cytoplasmatic targets for active metabolites) or the inhibition of microtubules in mitotic spindle assembly by protein disulfide bond formation after glutathione oxidation[21]. Thiola association with B(a)P stopped the c-mitosis formation in HDC, proving the involvement of non-protein sulfhydryls in the detoxication metabolism. Like all thiol compounds, Th reduces especially chromosome breaks and the formation of dicentrics and ring chromosomes.

Previously reported results revealed the modification of cellular response to many chemical carcinogens by thiols and thiol reagents[22–24], by scavenging the reactive species of mutagens/carcinogens[25–27], and preventing their reaching critical target sites of the cell, i.e. DNA, RNA and protein.

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