Protective Effect of the Antioxidant Stobadine Against Cyclophosphamide and Irradiation Induced Oxidative Stress

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Abstract. The antioxidant stobadine was tested for its efficiency against oxidative stress in model experiments with ICR nonpregnant mice exposed either to cyclophosphamide (80 mg/kg) or whole body ^{60}Co (6.5 Gy) irradiation. In a teratological experiment, pregnant mice were exposed to cyclophosphamide (10 mg/kg) from day 11 to 17 of gestation. Toxicity was measured by determining the lysosomal enzymes acid phosphatase and N-acetyl-β-D-glucosaminidase. Cyclophosphamide and irradiation caused a significant increase in acid phosphatase and N-acetyl-β-D-glucosaminidase activity in the spleen of nonpregnant mice. In the liver, lysosomal enzyme activities were unchanged and no changes in protein levels were recorded. In pregnant mice, acid phosphatase and N-acetyl-β-D-glucosaminidase activities were increased in the spleen. An increase in foetal acid phosphatase liver activity was found. Pretreatment with stobadine prior to cyclophosphamide and irradiation significantly diminished the biochemical changes in both nonpregnant and pregnant mice. We conclude that stobadine is able to protect mice against cyclophosphamide- or irradiation-induced oxidative stress.

Key words: Stobadine — Oxidative stress — Lysosomal enzymes — Cyclophosphamide — Irradiation

Introduction

Oxidative stress is defined as an imbalance between the production and inactivation of oxygen free radicals in cells with an accumulation of highly aggressive reactive oxygen species, which may lead to lipid peroxidation and protein cross-linking in cell membranes as well as to DNA damage resulting in cell destruction (Brogaard and Clausen 1997). This pathology can be treated by antioxidants (free radical scavengers), known to be effective in treating conditions associated with oxidative stress (Brogaard and Clausen 1997; Sun et al. 1998). Stobadine (STB) was proved to be a neuro- and cardio-protective drug with high free radical scavenging

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capacity (Orviský et al. 1997; Horáková and Štolc 1998; Mojžiš et al. 1998). Preclinical safety evaluation of STB was performed in rats and no adverse effects were detected (Gajdošíková et al. 1995). The present study investigates its effects in mice using two models of oxidative stress, namely high doses of cyclophosphamide and whole body ^{60}Co (6.5 Gy) irradiation. As disruption of lysosomal membrane integrity and leakage of lysosomal enzymes is one of the underlying processes of cell disintegration in oxidative stress (Zhang et al. 1992; Öllinger and Brunk 1995), the activity of lysosomal enzymes, acid phosphatase (APH) and N-acetyl-β-D-glucosaminidase (NAGA) in the spleen and foetal liver were used as markers of cell damage.

Materials and Methods

Animals ICR virgin female mice from the Breeding Facility IEP SASc, Dobrá Voda, Slovakia, were used The animals were kept under controlled conditions at 22±2°C and 55±5% relative humidity Food and tap water were available *ad libitum*

Drugs Stobadine (STB), cis-(-)-2,3,4,4a,5,9b-hexahydro-2,8-dimethyl-1H-pyrido[4,3b]indole, CAS No 95751-51-2 (Chemical Abstracts, 1987) in the form of dipalmitate salt (DP1031, M w 715 2, 99 5% purity) was developed in the IEP SASc, Bratislava, Slovakia (Štolc et al 1983) Its chemicophysical properties were described by Beneš and Štolc (1989) STB was dissolved in 0 5% methylcellulose (MC – Methocell MC 4000 cP, Fluka AG, Bush SG, Switzerland) Cyclophosphamide (CP) – Cyclostine® 200 (Pharmitalia Carlo Erba GmbH, Italy) All chemicals and enzyme substrates from Sigma, USA, were of analytical grade

Doses STB concentrations (23 60 and 70 07 mg/kg) were chosen according to a chronic 26-week toxicity study (Gajdošíková et al 1995) Dosage volume was 0 1 ml/10 g body weight

Experimental protocol

Experiment 1: Nonpregnant female mice (28–32 g, 8–12 weeks old, $n = 10$) were treated twice (24-hour interval) with CP in the dose of 80 mg/kg body weight intraperitoneally (i p) STB suspended in MC was administered orally (p o) in the concentration of 23 60 mg/kg body weight 2 h prior to or 4 h after CP administration, or in combination of the two treatments – 2 h prior and 4 h after CP (Chorvatovičová and Bauer 1994) Control animals were treated either with MC or STB The animals were sacrificed by cervical dislocation 24 h after the second CP injection

Experiment 2: Female mice (28–32 g, 8–12 weeks old) were divided into 6 groups Two of them were non irradiated controls, one treated with MC, the second with STB (70 07 mg/kg, p o) Four groups of mice were exposed to 6 5 Gy total body irradiation (Chisobalt cobalt-60 γ-ray source, National Institute of Oncology, Bratislava, Slovakia) One irradiated group was treated with MC, the other three groups with STB, one of them 2 h and the second 1 h prior to ^{60}Co, while the third immediately after irradiation The animals were sacrificed by cervical dislocation 40 h after irradiation

Experiment 3: Pregnant mice (initial weight 23–25 g, 8–10 weeks old) were used STB dissolved in MC was administered p o from day 11 until day 17 of gestation in the dose of 70 07 mg/kg, 2 h prior to cyclophosphamide (CP, 10 mg/kg, i p) On day 18 of pregnancy the animals were sacrificed by cervical dislocation The peritoneal cavity and uterus were opened and live foetuses were evaluated

In all three experiments, the spleen and foetal liver were removed, samples (50–60 mg) were put in ice cold phosphate buffer, pH 7 4, containing Triton X-100 (0 1%) and homogenised in a hand glass homogeniser Homogenates were centrifuged at 15,000 × *g* for 20 min As markers of cell damage, activity of the lysosomal enzymes acid phosphatase (APH) and N-acetyl-β-D-glucosaminidase (NAGA) were assayed according to standard methods (Barrett and Heath 1977) in supernatants after 15,000 × *g*

Statistical evaluation Student's *t*-test was used for statistical analysis

Results

Experiment 1 CP in the dose of 80 mg/kg induced a significant increase in APH and NAGA activity in the spleen (Fig. 1). Pretreatment with STB (23.60 mg/kg) 2 h prior to CP significantly inhibited the biochemical changes induced STB administered 4h after CP had no protective effect A combination of the two treatments

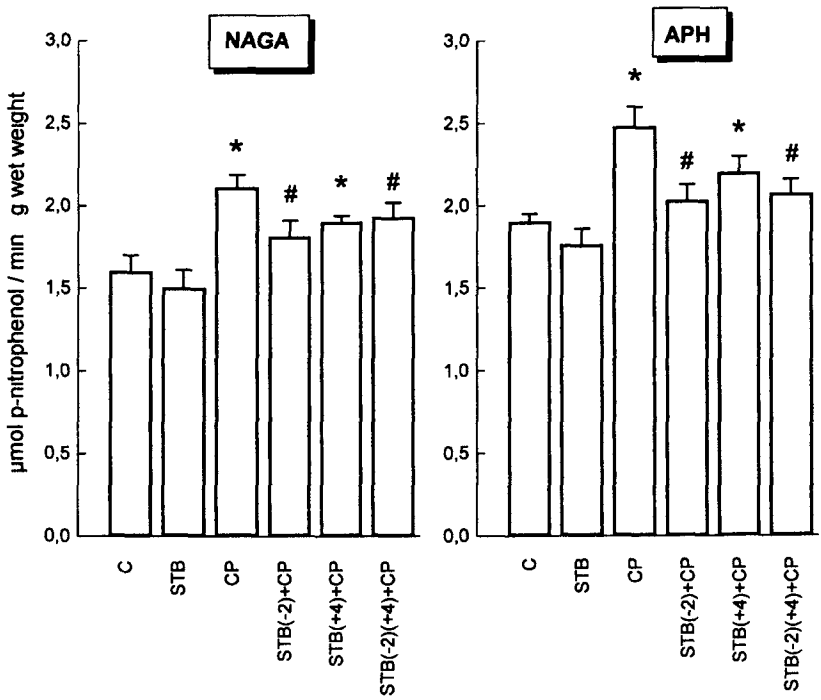

Figure 1. Effect of stobadine (STB) on cyclophosphamide (CP)-induced changes of lysosomal enzyme activities in the spleen C – Control, STB – STB (23 6 mg/kg), CP – CP (80 mg/kg), STB(−2)+CP – STB 2 h prior to CP, STB(+4)+CP – STB 4 h after CP, STB(−2,+4)+CP – STB 2 h prior and 4 h after CP NAGA – N-acetyl-β-D-glucosaminidase, APH – acid phosphatase Results are given as means ± S E M, ($n = 10$) Activity of NAGA and APH are expressed in μmol *p*-nitrophenol/min g wet weight *$p < 0\ 05$ versus control, #$p < 0\ 05$ versus CP

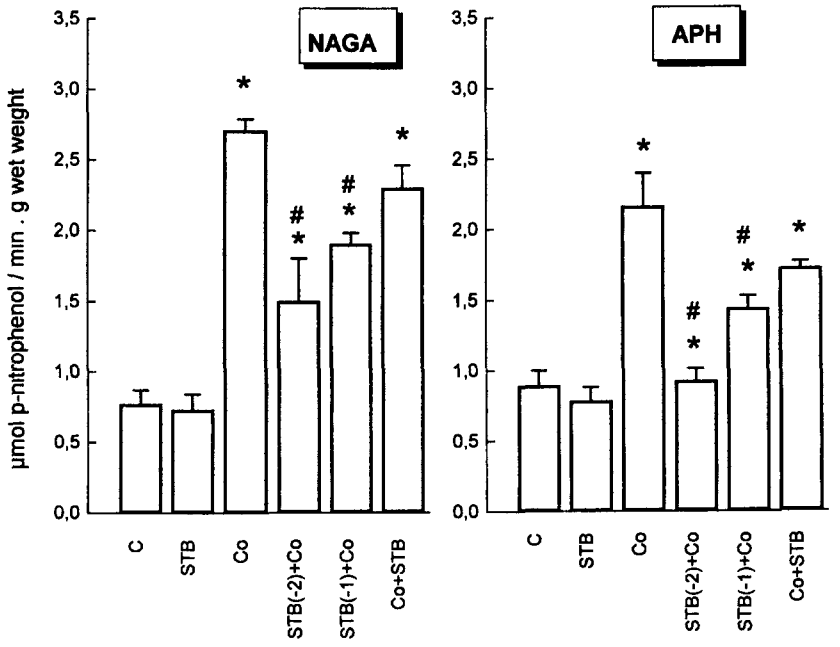

Figure 2. Effect of stobadine (STB) on irradiation (^{60}Co, 6 5 Gy)-induced changes of lysosomal enzyme activities in the spleen C – Control, STB – STB (70 07 mg/kg), Co – ^{60}Co (6 5 Gy), STB(-2)+Co – STB 2 h prior to ^{60}Co, STB(-1)+Co – STB 1 h prior to ^{60}Co, Co+STB – STB immediately after ^{60}Co NAGA – N-acetyl-β-D-glucosaminidase, APH – acid phosphatase Results are given as means ± S E M, (n = 10) Activity of NAGA and APH are expressed in μmol p-nitrophenol/min g wet weight *$p < 0$ 05 versus control, #$p < 0$ 05 versus CP

– 2 h prior and 4 h after CP – resulted in the same decrease as with STB 2 h prior to CP (Fig. 1).

Experiment 2· Irradiation of mice with ^{60}Co increased significantly the activity of NAGA and APH in the spleen. Oral pretreatment with STB (70.07 mg/kg) 2 h prior to ^{60}Co reduced the changes compared to irradiated controls (Fig. 2). The effect was lower when STB was administered 1 h prior to irradiation. Treatment with STB immediately after irradiation did not induce a statistically significant decrease of NAGA and APH activity (Fig. 2).

Experiment 3· In this experiment, the CP-induced toxic damage was associated with an increase in the maternal activities of APH and NAGA in the spleen (Fig. 3) and with an increase in foetal APH liver activity (Fig. 4). Pretreatment with STB partially inhibited the CP-induced increase of lysosomal enzyme activities in maternal spleen and of APH activity in foetal liver (Figs. 3 and 4).

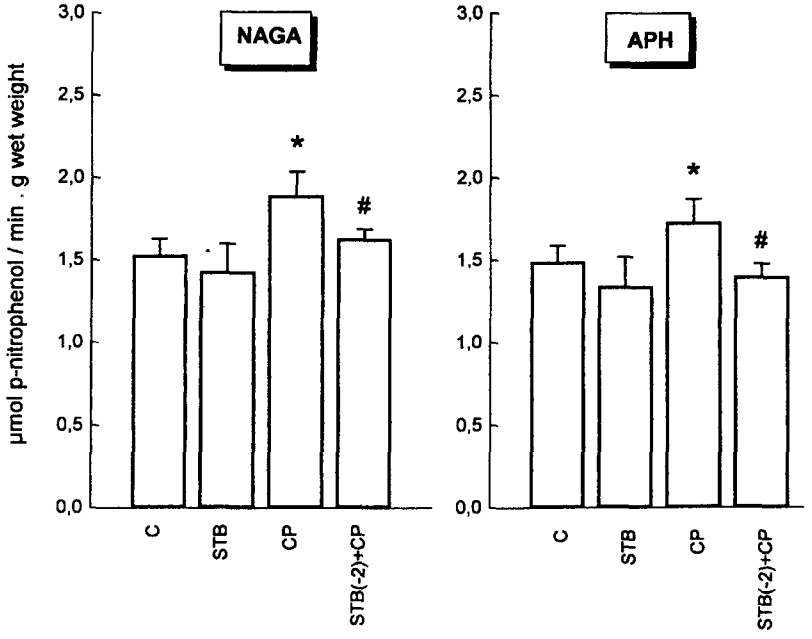

Figure 3. Effect of stobadine (STB) on cyclophosphamide (CP)-induced changes of lysosomal enzyme activities in maternal spleen. C – Control; STB (-2) – STB (70.07 mg/kg); CP – CP (10 mg/kg); STB(-2)+CP – STB 2 h prior to CP. NAGA – N-acetyl-β-D-glucosaminidase; APH – acid phosphatase. Results are given as means ± S.E.M, (n = 10). Activity of NAGA and APH are expressed in μmol p-nitrophenol/min.g wet weight. $^*p < 0.05$ versus control, $\#p < 0.05$ versus CP.

Discussion

Cyclophosphamide (CP) is one of the most widely used antitumour and immunosuppressant drugs. Metabolisation of CP in organisms resulted in free radical generation (Kanekal and Kehrer 1994). Radiation was shown to enhance the production of reactive oxygen species in a variety of cells (Alaoui et al. 1992; Sun et al. 1998). The reactive oxygen species, which are formed during exposure of cells to CP or ionising radiation, are able to kill tumour cells by causing damage to DNA, membranes and enzymes. Increased intracellular concentration of reactive oxygen metabolites may be harmful to the integrity of secondary lysosomes because they constitute a compartment where iron would exist as a low-molecular weight complex with the ability to support peroxidation and fragmentation reactions (Brunk et al. 1995). Lysosomal destabilisation may be prevented either by inhibition of cellular peroxidation or by prevention of iron-catalysed oxidative reactions, which involve peroxidation of cellular membranes, energy depletion, and leakage of lysosomal content. Understanding of the biochemical and molecular changes associated

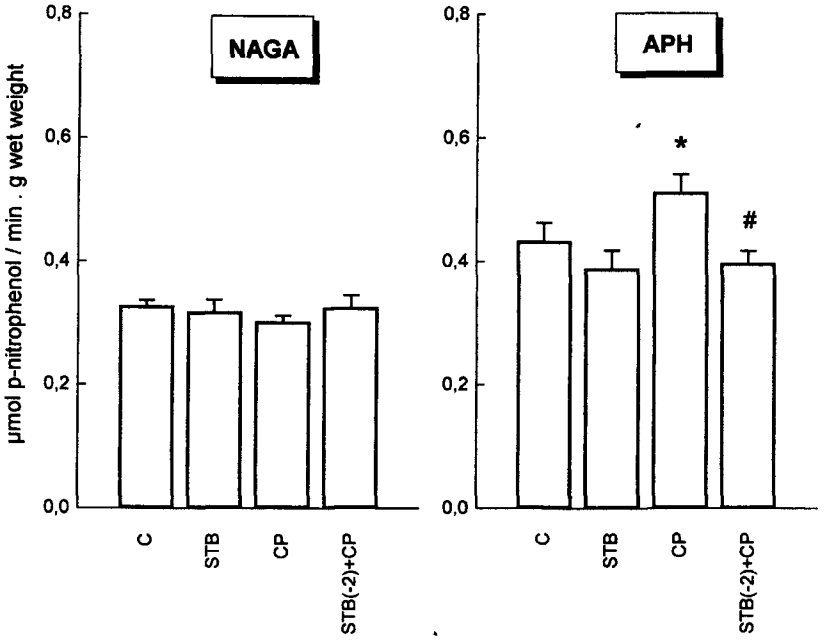

Figure 4. Effect of stobadine (STB) on cyclophosphamide (CP)-induced changes of lysosomal enzyme activities in foetal liver. C – Control; STB – STB (70.07 mg/kg); CP – CP (10 mg/kg); STB(-2)+CP – STB 2 h prior to CP. NAGA – N-acetyl-β-D-glucosaminidase; APH – acid phosphatase. Results are given as means ± S.E.M, (n = 10). Activity of NAGA and APH are expressed in μmol p-nitrophenol/min.g wet weight. $^*p < 0.05$ versus control, $\#p < 0.05$ versus CP.

with oxidative stress may promote establishment of experimental models for testing drugs protecting tissues from injury (Kehrer and Lund 1994). Oxidative stress which damages cells through disruption of lysosomal integrity (Zhang et al. 1992; Öllinger and Brunk 1995) can be reduced with antioxidants (Brogaard and Clausen 1997; Sulkowska et al. 1998; Sun et al. 1998).

In our previous study (Navarová et al. 1999), we reported that the antioxidant STB was found to be an efficient inhibitor of lysosomal enzyme release *in vivo* on whole animals and that this effect was comparable with its *in vitro* effect on HeLa cells in tissue culture.

In the present study, we showed that irradiation and CP caused marked changes in the activity of lysosomal enzymes (NAGA and APH) and that pretreatment with STB inhibited these biochemical changes, yet administered therapeutically it failed to exert its effect. The same is known about its antimutagenic effects (Chorvatovičová and Bauer 1994). STB may conceivably be effective in the initial step of the oxidative stress-induced biochemical changes.

Shogi and Ohzu (1965) were first to report on the teratogenicity of CP in pregnant mice The most sensitive period in terms of CP-induced defects is the period from day 10 through day 12 of gestation (Gibson and Becker 1968). In whole embryos developing either *in vivo* or *in vitro,* teratogen-induced oxidative stress has been linked to dismorphogenesis that results from exposure to a number of agents or exogenous conditons (Kotch et al. 1995). In our previous experiment, we reported that administration of CP from day 11 to day 17 of gestation resulted in decrease of foetal weight and CP induced exophthalmos, cleft palate, limb malformations, brachycaudia, tail haematoma and hydrops abdominis (Navarová et al 1995). It is suggested that dismorphogenesis relates to the changing biochemistry and physiology of the conceptus.

STB is an efficient scavenger of hydroxyl radicals (Štefek and Beneš 1991; Horáková et al. 1994) and thus its scavenging potential may be the mechanism underlying the protective effect established in our experiments From results presented in this study we conclude that STB is able to protect mice against CP- or irradiation-induced oxidative stress.

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