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NITRIC OXIDE SYNTHASE INHIBITORS REDUCED LIPID PEROXIDATION IN *N*-NITROSODIETHYLAMINE-TREATED RATS

INHIBITORY SYNTAZY TLENKU AZOTU REDUKOWAŁY PEROKSYDACJĘ LIPIDÓW U SZCZURÓW ZATRUWANYCH N-NITROZODIMETYLOAMINĄ

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Present studies elucidate the anti-oxidative effectiveness of nitric oxide synthase (NOS) inhibitors including L-N$^{\omega}$-nitro-L-arginine methyl ester and spermidine in rats pretreated with N-nitrosodiethylamine.

INTRODUCTION

The involvement of lipid peroxidation in *N*-nitrosodiethylamine (NDEA)-induced liver toxicity and/or carcinogenicity has been well documented [3, 4, 9], however, little is know about the role of nitric oxide (NO) and other nitrogen reactive species in these pathologies. Previously, it was suggested that NO and its red-ox derivatives such as peroxynitrite ($ONOO^-$) is elevated in inflammation processes and play an important role in liver carcinogenesis [13, 16, 22]. It should be emphasized that human hepatocellular carcinomas were usually associated with chronic cirrhosis and/or inflammation such those caused by viruses (viral hepatitis B and C) [8]. Therefore, it was of interested to note that the expression of the inducible isoform of nitric oxide synthase (iNOS) and 3-nitrotyrosine, a marker of peroxynitrite production in preneoplastic and/or neoplastic rat liver tissues has been also induced by NDEA [1].

Since peroxynitrite is an endogenous oxidizing and nitrating agent as well as modulator of liver toxicity, and nitric oxide (NO) may acts both as a pro-oxidant and/or anti-oxidant agent in hepatocytes [2, 6, 16, 18], an intriguing aspect of the present studies was the treatment of NDEA-poisoned rats with NOS inhibitors. In these experiments we tried to gain further insight into the effect(s) of *L*-N$^{\omega}$-nitro-L-arginine methyl ester (L-NAME) on the level of lipid peroxidation and/or pro-oxidant shift(s) in NDEA-treated rats. Since polyamines have been originally produced from the L-arginine pathway *via* the formation of L-ornithine, a fist-step agent in putrescine biosynthesis [12], the possibility that spermidine, a simple polyamine produced from putrescine may decreases lipid peroxidation in rats was also investigated.

MATERIAL AND METHODS

Male *Wistar* rats (220 ± 20 g) were used in the studies. Before the experiment, the animals were acclimatized for two weeks under standard conditions (ambient temperature 22±2°C, air humidity 40–70%, light-darkness cycle 12/12 h). Throughout the experiment, the rats were given a standard laboratory chow (Murigran pellet, Motycz, Poland) and water *ad libitum*. The animals were divided into 4 groups of 8–10 rats in each group, and they were treated *per os* with saline – control (group 1), spermidine 10 mg/kg b.w. (group 2), *N*-nitrosodiethylamine (NDEA) 0.1 mg/kg b.w. (group 3), or NDEA 0.1 mg/kg b.w. plus spermidine 10 mg/kg b.w. (group 4) daily for 30 days. In the group 2 and 4, spermidine was dosed only for 21 days, and the agent was introduced at 3–4 hrs after pretreatment with saline or NDEA, respectively. On day 22nd of the experimental period, the half of randomly selected rats in each group were treated *per os* with *L*-N^{ω}-nitro-L-arginine methyl ester (L-NAME) 10 mg/kg b.w. daily for 3 days. At 24 hrs after the last pretreatment with saline or NDEA (day 31st), the animals were sacrificed by cervical dislocation and thiobarbituric-acid reactive substances (TBARS) were determined in the small intestine mucosa, liver, spleen, and kidney by the method of *Ohkawa* et al. [21]. Briefly, thiobarbituric acid test was performed using 100 μl of 10% tissue homogenate (prepared in 1,15% KCl), which was added to 100 μl of 8,1% SDS. Thereafter, 20% glacial acetic acid and 2-thiobarbituric acid (v/v) were added to the reaction mixture. To start the reaction, the samples were heated for one hour at 95°C, and then were cooled in a water bath. The mixtures were extracted with a spectral pure *n*-butanol and centrifuged (4000,0 x g) at 4°C for 10 minute. All butanol extracts were measured spectrophotometrically at 532 nm. Standard samples contained 1,1,3,3-tetraethoxypropane instead of homogenate. Protein content was measured by the method of Lowry et al. [17] with bovine serum albumin as a standard. All reagents for a dosage were of the highest quality available and purchased from Merck (Darmstadt, Germany) and Sigma Chemical Company (St. Louis, MO, USA) and dissolved in a double-distilled water.

The statistical significance of the differences was determined by using *Student's t*-test for comparison between the repeated measures analysis of variance (ANOVA) in two groups and *Dunnet's* tests for multiple comparison where appropriate. Differences were considered significant when probability (p) values were less than 0.05.

RESULTS AND DISCUSSION

The main findings of these studies shown that NDEA increased lipid peroxidation in the small intestine and liver of rats, however, the agent did not have any effects in the kidney and spleen, respectively (Fig. 1). Pretreatment of rats with spermidine decreased NDEA-induced TBARS in the small intestine and liver, but this polyamine did not elucidate any further effect(s) in the rest of tissues and those animals treated with saline (Fig. 1). In accordance with our results, polyamines including putrescine, spermidine, and spermine have been shown to protect DNA in vitro against radiation-induced oxygen radicals [26]. It should be noted that other biogenic amines such as tryptamine and tyramine also inhibited the oxidation of linoleic acid in vitro [28]. In addition to the inhibition of lipid peroxidation in rodent tissues, *Grudziński et al.* [10, 11] have evidenced that putrescine is enable to decrease sodium nitrite-induced TBARS in the small intestinal mucosa of rats. Since L-arginine has been found to be a major amino acid in polyamine biosynthesis *via* L-ornithine in cells [12], we have decided to examine the anti-oxidant activity of some L-arginine derivatives against lipid peroxidation and/or pro-oxidant shift(s) in NDEA-treated rats. Among the nitro-*L*-arginine analogues, *L*-N^{ω}-nitro-L-arginine methyl ester (L-NAME) has been frequently employed in the present studies.

Fig. 1. The effect of L-NAME and spermidyne on the level of thiobarbituric acid reactive substances (TBARS) in the small intestinal mucosa, liver, kidney, and spleen of *N*-nitrosodiethylamine-treated *Wistar* rats.
Open and filled bars represent groups without or with L-NAME, respectively. (Control) saline, (SP) spermidine, (NDEA) *N*-nitrosodiethylamine, (NDEA+SP) *N*-nitrosodiethylamine plus spermidine. Values are mean ± SEM, n = 8–10. * P < 0.05, NDEA *vs*. saline, ** P < 0.05, NDEA *vs*. NDEA plus spermidyne, *** P < 0.05, NDEA or NDEA plus spermidine *vs*. NDEA plus L-NAME or NDEA plus spermidine plus L-NAME, respectively.

Results of these experiments show that L-NAME decreases TBARS in the liver and small intestine of NDEA-treated rats (Fig. 1). However, in animals pretreated with NDEA and spermidine, the inhibitory effect(s) of L-NAME was only observed in the small intestinal mucosa (Fig. 1). It should be emphasized that both L-NAME and spermidine also mitigated lipid peroxidation in the small intestine of rats pretreated with *N*-nitrosomethylurea for 30 days (data not shown). Since NDEA elevated both iNOS enzyme and 3-nitrotyrosine, a marker of peroxynitrite formation in rat liver tissues [1], the present results are in agreement with those reported by others [1, 5], suggesting that L-NAME might elucidate some anti-oxidative properties against peroxynitrite-mediated pro-oxidant shift(s). In many instances, it is becoming apparent that the peroxynitrite ion ($ONOO^-$) and/or either nitrite (NO_2^-) or nitrate (NO_3^-), the end-product(s) of peroxynitrite decomposition in cells, serves as a mediator(s) in oxidative action originally attributed to $NO^{\cdot}$ and/or other oxygen-derived reactive species [23, 27]. Since we have found that the inhibition of NOS enzyme protects against lipid peroxidation in NDEA-treated rats, it was suggested that $NO^{\cdot}$ and/or peroxynitrite might be involved in NDEA-induced pro-oxidant shift(s) (Fig. 1). Although L-NAME has shown beneficial effect(s) against the toxicity of some chemicals, e.g. sulfur mustard [24], those effects are probably not responsible for the action(s) of NDEA in rat liver, because there is also other evidence that NOS inhibition by L-NAME increases NDEA-induced liver injury in rats [20]. With precaution it should be noted that L-NAME was found to increase TBARS in the liver of rats pretreated with carbon tetrachloride, further indicating that nitric oxide (NO) also possessed some anti-oxidative properties [19].

It is now widely accepted that nitric oxide (NO) might play a cytoprotective role by acting as anti-oxidant agent [15]. On the other hand, the overproduction of NO from L-arginine, and the cellular formation of peroxynitrite (a diffusion-controlled reaction of $NO^{\cdot}$ and superoxide radicals), have been recently implicated as a major cause of liver lesions, thus contributing to preneoplastic and/or neoplastic changes in NDEA-treated rats [1]. Since liver pathologies may result from unwanted induction NOS enzyme by pro-inflamatory cytokines such as tumor necrosis factor, interleukins, and bacterial lipopolysacharide [7, 18, 25], much attention has been paid to examine NOS inhibitors specific for NOS II isozyme. Our studies preliminary indicated that L-NAME and spermidine could be an anti-oxidant agent(s) in NDEA-induced lipid peroxidation. Since the oxidation and/or nitration of tyrosine residue(s) by peroxynitrite disrupts the post-translational modifications of proteins [14], the present finding require further investigation to elucidate the protective activity of NOS inhibitors against the formation of DNA adducts. Therefore, 8-nitroguanine and/or 8-oxoguanine should be monitored as a specific marker(s) for peroxynitrite-mediated DNA damage in NDEA-treated animals.

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Summary

Studies were carried out to examine the anti-oxidative activity of nitric oxide synthase (NOS) inhibitors including *L*-N$^{\omega}$-nitro-L-arginine methyl ester (L-NAME) and spermidine in rats pretreated *per os* with *N*-nitrosodiethylamine (NDEA) (0.1 mg/kg b.w.) daily for 30 days. Both L-NAME and/or sapermidine were gavaged to animals in a daily dosage of 10 mg/kg body weight, however, the polyamine was applied for the first 21 days only, and further L-NAME was employed for 3 days (day 22, 23 and 24). Saline treated rats were served as control. The results of this experiment showed that NDEA increased TBARS in the liver and small intestine of rats, and the agent did not have any effect(s) in spleen and kidney, respectively. Pretreatment of animals with spermidine and/or L-NAME significantly ($p<0.05$) lowered lipid peroxidation in NDEA-treated rats. The finding described here elucidate that both L-NAME and spermidine play an important anti-oxidative role in NDEA-mediated lipid peroxidation and/or pro-oxidant shift(s) in rats.

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Streszczenie

W pracy oceniano anty-oksydacyjne właściwości inhibitorów syntazy tlenku azotu (NO) tj. estru metylowego nitro-L-argininy (L-NAME) oraz spermidyny poprzez pomiary ilości substancji reagujących z kwasem tiobarbiturowym u szczurów zatruwanych *per os N*-nitrozodiethyloaminą (NDEA) (0.1 mg/kg m.c./dzień) przez okres 30 dni. Inhibitory podawano *per os* w dawce 10 mg/kg m.c./dzień, przy czym spermidinę podawano przez pierwsze 21 dni zatrucia, natomiast L-NAME tylko w 22, 23 i 24 dniu eksperymentu. Szczury kontrolne otrzymywały *per os* 0.9% roztwór chlorku sodowego. W przeprowadzonych badaniach wykazano pro-oksydacyjne właściwości NDEA w wątrobie oraz błonie śluzowej jelita cienkiego, przy braku takiego oddziaływania w śledzionie oraz nerkach. Inhibitory syntazy tlenku azotu (L-NAME i spermidina) obniżały poziom peroksydacji lipidowej indukowanej przez NDEA, wykazując anty-oksydacyjne właściwości w przebiegu 30-dniowego zatrucia szczurów *N*-nitrozodietyloaminą.

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