ROCZN. PZH, 2002, 53, NR 1

IRENEUSZ P. GRUDZIŃSKI, ANNA FRANKIEWICZ-JÓŹKO

FURTHER STUDIES ON THE ANTI-OXIDATIVE EFFECT OF PUTRESCINE IN SODIUM NITRITE-TREATED RATS

DALSZE BADANIA NAD ANTYOKSYDACYJNYM WPŁYWEM PUTRESCYNY U SZCZURÓW ZATRUWANYCH AZOTYNEM SODOWYM

Department of Applied Physiology
Military Institute of Hygiene and Epidemiology
Kozielska 4, 01–163 Warsaw, Poland
Head: prof. dr hab. med. *J. Faff*

In recent years, dietary polyamines, including putrescine, have attracted considerable interest because of the suggestion that their presence in human diet may have some anti-oxidative properties. Therefore, studies were carried out to elucidate the anti-oxidative effect(s) of oral putrescine supplementation in rats pretreated per os with either sodium nitrite or normal saline (control). Results suggest that putrescine is an effective anti-oxidant agent, which mitigates nitrite-induced lipid peroxidation in rat liver and small intestinal mucosa.

INTRODUCTION

The polyamines, putrescine, spermidine, and spermine are ubiquitous compounds of all mammalian cells, and are involved in a variety of regulatory steps of protein and/or nucleic acid biosynthesis [10]. It is generally accepted that polyamines play a pivotal role in cellular growth and/or proliferation processes [25], however a large body of experimental evidences also exists that polyamines act as potent anti-oxidants. For example, putrescine has been found to suppress the production of lipid peroxides and promote DNA synthesis in liver regeneration after ischemia-reperfusion injury [20]. In other recent report, the anti-oxidative and anti-inflammatory effect(s) of spermine was discussed [15]. It should be noted that spermine was found to prevent lipid peroxidation induced by essential fatty acids in human breast cancer cells [4], and the agent also inhibited the Fe(III)/xanthine oxidase stimulated lipid peroxidation of brain phospholipid liposomes [14]. As evidenced by *Awasthi* and associates [1], spermidine has been shown to inhibit the *in vitro* formation of thiobarbituric acid-reactive substances (TBARS) from sonicated vesicles of rat brain. Putrescine, spermidine and spermine have been also noted to decrease paraquat-induced augmentation of lipid peroxidation and superoxide dismutase activity in the lungs of rats [11]. It should be emphasized that polyamines were evidenced to protect phi X-174 plasmid DNA from strand breakage promoted by reactive oxygen species [9], and polyamine-lowering drug-candidates have been recently tested in anti-cancer therapy [23].

Inorganic nitrites occur widely in human diet and drinking water, both as intentional additives and as undesirable contaminants [13]. Since nitrites have been recognized as a risk factor(s) of gastric and/or colorectal cancers in humans and animals [5–7, 12], the major goal of this study was to evidence that a short-term oral putrescine supplementation could mitigate sodium nitrite-induced lipid peroxidation and/or pro-oxidant shift(s). The total anti-oxidant status (TAS) of rat blood and the activity of Cu,Zn-superoxide dismutase (SOD) was also examined in sodium nitrite-poisoned rats and supplemented with or without putrescine.

MATERIALS 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. Throughout the experiment, the rats were given a standard laboratory chow (Murigran, Motycz, Poland) and water *ad libitum*. The animals were divided into 2 groups of 14 rats in each group, and they were treated *per os* with either an aqueous solution of sodium nitrite (10 mg/kg b.w) or normal saline (control) daily for 14 days. On day 7th of the experimental period, the half of randomly selected nitrite – or saline-treated rats was pretreated *per os* with putrescine (10 mg/kg b.w) for 7 days only. The agent was dissolved in normal saline and daily dosed to rats at 3–4 hr post-nitrite and/or post-saline pretreatment. The animals were sacrificed by cervical dislocation 24 hr after the last nitrite and/or saline dosage (day 15) and the total antioxidant status (TAS) of rat blood and thiobarbituric acid reactive substances (TBARS) in serum and the small intestinal mucosa and/or liver samples were determined. The activity of Cu,Zn-superoxide dismutase (EC 1.15.1.1) was also assayed in selected tissues.

Thiobarbituric acid test was employed according to the method described by *Ohkawa* and co-workers [21]. Briefly, samples of 100 µl of rat serum and/or 100 µl of 10% tissue homogenates (prepared in 1.15% KCl) was added to 100 µl of 8.1% SDS. Thereafter, a solution of 20% glacial acetic acid and 0.8% 2-thiobarbituric acid (v/v) was added to the reaction. To start the reaction, the samples were heated for one hour at 95^{o}C, and then were cooled in a water bath. The mixture were extracted with a spectral pure *n*-butanol and centrifuged (4000 x g) for 10 min at 4^{o}C. All butanol extracts were measured spectrophotometrically at 532 nm. Standard samples contained 1,1,3,3-tetraethoxypropane instead of homogenate.

The total antioxidant status of rat blood was determined using the diagnostic RANTAS^{TM} assay kit (Radnox Laboratories Ltd., Antrim, UK). The assay was employed as described in details by the TAS manual protocol (RANTAS^{TM}, Randox, 1993, pp. 1–6). Briefly, the azo-compound ABTS (2,2'-Azino-di-[3-ethylbenzthiazoline sulphonate]) was incubated with a peroxidase (metmyoglobin) and hydrogen peroxide (H_2O_2) to produce the radical cation $ABTS^{\cdot+}$. The cation has had a relatively stable blue-green colour, which it was measured spectrophotometrically at 600 nm. Antioxidants in the added sample (rat serum) caused suppression of this colour production to a degree, which was proportional to their concentration. In the present assay, TMCA® (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) was used as a standard.

The activity of Cu,Zn-superoxide dismutase (SOD) was assayed using the diagnostic RANSOD^{TM} assay kit (Radnox Laboratories Ltd., Antrim, UK). The assay was employed as described in details by the SOD manual protocol (RANSOD^{TM}, Randox, 1997, pp. 1–7). In this method, both xanthine and xanthine oxidase was employed to generate superoxide radicals, which further react with 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium chloride to form a red formazan dye. The superoxide dismutase activity was measured specrophotometrically by the degree of inhibition of this reaction at 505 nm. In the present assay, the whole blood samples were centrifuged (2000 x g) for 10 min at 4^{o}C and washed with 0.9% NaCl for three times, and further lysed in a total volume of 2 ml of ice-cold double distilled water. The lysate was diluted

with 0.01 mmol/l phosphate buffer, pH 7.0, so that the percentage of inhibition of reaction falls between 30% and 60%. The small intestinal mucosa and/or liver tissues were homogenized in a solution of 1.15% KCl to yield 10% homogenates, which were centrifuged (600 x g) for 10 min at 4°C. The supernatants were centrifuged (15000 x g) for 15 min at 4°C, and the post-mitochondrial supernatant(s) was diluted with 0.01 mmol/l phosphate buffer, pH 7.0 as mentioned above. In the present studies, a 20-fold, 200-fold, or 200-fold dilution of samples was recommended for the rat small intestinal mucosa, liver or blood, respectively. Protein content was measured by the method of *Lowry* et al. [16] with bovine serum albumin as a standard. The whole blood haemoglobin was also estimated by the Reflotron stripe test (Roche, Basal, Switzerland). All reagents were of the highest quality available from Randox Laboratories Ltd., (Antrim, UK) and Sigma Chemical Company (St. Louis, MO, USA).

The results were subjected to statistical analysis by *Student's t*-test for unpaired samples. Differences were considered significant when probability (p) values were less than 0.05.

RESULTS AND DISCUSSION

Sodium nitrite has been shown to increase thiobarbituric acid reactive substances (TBARS) in the small intestinal mucosa and liver of rats and the agent did not have any effect on the total anti-oxidant status (TAS) and lipid peroxidation of rat blood (Figs. 1, 2). As shown in figure 2, sodium nitrite did not change the activity of SOD in the small intestinal mucosa, liver, and/or blood respectively. In the present studies, however, the nitrite dosage did not affect the level of haemoglobin in rat blood samples (Fig. 1). It should be noted that sodium nitrite has been recently found to elevate lipid peroxidation in red cell membranes, and the agent also generated reactive nitrogen species such as peroxynitrite ($ONOO^-$) in human bronchial epithelial cells *in vitro* [28, 30]. In our previous experiments, sodium nitrite has been shown to increase the activity of ornithine decarboxylase [8], a first-step enzyme in polyamine biosynthesis. Although putrescine has been mainly accredited to be intracellular synthesized molecule, it is also derived to human and/or animal body from other extra-cellular sources, particularly the diet (meat, fruit, cheese and non-green vegetables) and bacterial resident in the gastrointestinal tract is increasingly recognized [2, 3]. In recent years, therefore, dietary polyamines including putrescine, spermidine, and/or spermine have attracted considerable interest because of the suggestion that their presence in human diet may have some anti-oxidative properties.

Pretreatment of nitrite-poisoned rats with a daily dose of putrescine (10 mg/kg b.w) decreased the amount of TBARS in examined tissues (Fig. 1). Interestingly, putrescine enhanced the level of the total anti-oxidant status of blood in animals treated with or without sodium nitrite (Fig. 2). As shown in figure 2, putrescine also decreased SOD activity in rat blood and liver, however, it did not have any effect on the SOD enzyme in the small intestinal mucosa. These results were found in accordance with those previously reported by *Khanna* and associates [11], who showed that putrescine is enable to diminish both paraquat-induced TBARS and SOD activity in rat lung tissues. It should be noted that the beneficial effect(s) of dietary putrescine supplementation on carbon tetrachloride-and/or D-galactosamine-induced lipid peroxidation in rats was reported by *Nagoshi* and colleagues [18]. Similarly, *Mizui* and associates evidenced the protective mechanism of putrescine against ethanol-induced gastric lesions and lipid peroxidation [17]. In other recent studies, putrescine and spermidine have been found

to serve as a free-radical scavenger in mice [19]. It is of interested to note that the interaction of spermidine with hepatic microsomal lipids has been postulated to be responsible for the inhibition of lipid peroxidation in rat liver [22].

In conclusion, the present results show the anti-oxidative effect(s) of putrescine in the small intestinal mucosa and liver of sodium nitrite-treated rats. With precaution it should be noted that the polyamine also mitigated SOD activity in selected tissues. Although, the biological role of SOD is detoxification of superoxide radical to hydrogen peroxide and oxygen, the enzyme can also acts as a peroxidase in the presence of hydrogen peroxide, plausibly leading to inactivation of SOD [27]. Interestingly, superoxide dismutase has been also shown to react with putrescine to form a stable putrescine-SOD complex [24]. Since the oxidative degradation of endogenous polyamines including putrescine have been recently considered as a cause of apoptotic cell death in murine small intestine [26] and SOD enhanced oxidative DNA damages caused by cysteine/iron-catalyzed oxidation system [29], a clear effect(s) of the dietary polyamine load should be re-established in further studies.

I.P. Grudziński, A. Frankiewicz-Jóźko

FURTHER STUDIES ON THE ANTI-OXIDATIVE EFFECT OF PUTRESCINE IN SODIUM NITRITE-TREATED RATS

Summary

Studies were carried out to elucidate the anti-oxidative effect(s) of putrescine (10 mg/kg b.w./day) in rats treated *per os* with either sodium nitrite (10 mg/kg b.w./day) or normal saline (control) for 14 days. The putrescine was given to rats for 7 days only (days 7–14) and it was introduced 3–4 hrs after nitrite or saline dosage. Sodium nitrite increased thiobarbituric acid reactive substances (TBARS) in rat small intestinal mucosa and liver, and the agent did not have any effect on the total anti-oxidant status (TAS) and lipid peroxidation of rat blood. Nitrite did not also change the activity of superoxide dismutase (SOD) in the small intestinal mucosa, liver and blood, as well. Pretreatment of nitrite-treated rats with putrescine decreased TBARS and increased TAS in animals. Putrescine decreased SOD activity in the blood and liver of nitrite- and/or saline-treated rats, however, the agent did not affect the SOD enzyme in the small intestinal mucosa. Results suggest that putrescine dosed to nitrite-treated rats possesses some anti-oxidative properties.

I.P. Grudziński, A. Frankiewicz-Jóźko

DALSZE BADANIA NAD ANTYOKSYDACYJNYM WPŁYWEM PUTRESCYNY U SZCZURÓW ZATRUWANYCH AZOTYNEM SODOWYM

Streszczenie

Badano antyoksydacyjne właściwości putrescyny (10 mg/kg m.c./dzień) u szczurów zatruwanych *per os* azotynem sodowym (10 mg $NaNO_2$/kg m.c./dzień) przez okres 14 dni. Putrescynę podawano *per os* w 3–4 godz. po podaniu azotynu i/lub 0,9% NaCl (kontrola) przez okres 7 dni (dzień 7–14). Azotyn sodowy zwiększył poziom substancji reagujących z kwasem tiobarbiturowym (TBARS) w błonie śluzowej jelita cienkiego i wątrobie, nie wpływając na zmiany tego parametru we krwi szczurów. Nie wykazano, aby u zwierząt zatruwanych azotynem dochodziło do zmian zarówno stanu antyoksydacyjnego surowicy (TAS) oraz aktywności dysmutazy ponadtlenkowej (SOD) we krwi, wątrobie czy błonie śluzowej jelita cienkiego. Podawanie szczurom putrescyny

zmniejszyło poziom TBARS w badanych tkankach przy zwiększonym poziomie TAS w surowicy. Putrescyna znacząco obniżyła aktywność SOD w wątrobie i krwi nie zmieniając aktywności tego enzymu w jelicie cienkim. Rezultaty badań sugerują, że podawanie putrescyny *per os* szczurom może prowadzić do obniżenia peroksydacji lipidowej indukowanej azotynem sodowym.

REFERENCES

1. *Awasthi S., Kakkar P., Viswanathan P.N.*: Inhibition of liposomal lipid peroxidation by spermidine. J. Microencapsul. 1992, 9, 237–242.
2. *Bardocz S., Duguid T.J., Brown D.S., Grant G., Pusztal A., White A., Ralph A.*: The importance of dietary polyamines in cell regulation and growth. British J. Nutr. 1995, 73, 819–828.
3. *Bardocz S., Grant G., Brown D., Ralph A., Pusztai A.*: Polyamines in food – implications for growth and health. J. Nutr. Biochem. 1993, 4, 66–71.
4. *Chapman G.E., Wallace H.M.*: Spermine prevents lipid peroxidation induced by essential fatty acids in human breast cancer cells. Biochem. Soc. Trans. 1994, 22, 401-404.
5. *Eicholzer M., Gutzwiller F.*: Dietary nitrates, nitrites, and N-nitroso compounds and cancer risk: A review of the epidemiological evidence. Nutr. Res. 1998, 56, 95–105.
6. *Furukawa F., Nishikawa A., Ishiwata H., Takahashi M., Hayashi Y., Hirose M.*: Renal carcinogenicity of concurrently administered fish meal and sodium nitrite in F344 rats. Jpn. J. Cancer Res. 2000, 91, 139–147.
7. *Gatseva P.D., Mardirosian Z.H., Popova E.J., Iskrenova E.S., Vladeva S.V., Pavlova K.I.*: Evaluation of health hazards in children from regions with nitrate pollution. Folia Med. (Plovdiv) 2000, 42, 19–22.
8. *Grudziński I.P., Szymański A.*: Effect of sodium nitrite on gastric mucosa of rats: Ornithine decarboxylase activity. Polish J. Environ. Studies 1999, 8 (Supl. II), 274–276.
9. *Ha H.C., Yager J.D., Woster P.A., Casero R.A.*: Structural specificity of polyamines and polyamine analogues in the protection of DNA from strand breaks induced by reactive oxygen species. Biochem. Biophys. Res. Commun. 1998, 244, 298–303.
10. *Heby O., Person L.*: Molecular genetics of polyamine synthesis in eukaryotic cells. Trends Biochem. Sci. 1990, 15, 153–158.
11. *Khanna Y.P., Taneja S.K., Raj H.G., Venkitasubramanian T.A.*: Polyamines modify paraquat-induced changes in pulmonary superoxide dismutase and lipid peroxidation. Res. Commun. Chem. Pathol. Pharmacol. 1982, 35, 337–340.
12. *Knekt P., Jarviven R., Dich J., Hakulinen T.*: Risk of colorectal and other gastro-intestinal cancers after exposure to nitrate, nitrite, N-nitroso compounds: a follow-up study. Int. J. Cancer 1999, 80, 852–856.
13. *Korenekova B., Kottferova J., Korenek M.*: The fat of added nitrate used in the manufacture of Emmental cheese. Food Addit. Contam. 2000, 17, 373–377.
14. *Lovaas E., Carlin G.*: Spermine: an anti-oxidant and anti-inflammatory agent. Free Radical Biol. Med. 1991, 11, 455–461.
15. *Lovaas E.*: Hypothesis: spermine may be an important epidermal antioxidant. Med. Hypotheses 1995, 45, 59–67.
16. *Lowry G.H., Rosenbrough N.J., Farr A.L., Randal R.J.*: Protein measurements with the *Folin* phenol reagent. J. Biol. Chem. 1951, 193, 265–275.
17. *Mizui T., Shimono N., Doteuchi M.*: A possible mechanism of protection by polyamines against gastric damage induced by acidified ethanol in rats: polyamine protection may depend on its antiperoxidative properties. Jpn. J. Pharmacol. 1987, 44, 43–50.
18. *Nagoshi S., Ohta Y., Matsui A., Fujiwara K.*: Protective action of putrescine against rat liver injury. Scand. J. Gastroenterol. 1994, 29, 166–171.
19. *Nilsson J., Gritli-Linde A., Heby O.*: Skin fibroblasts from spermine synthase-deficient hemizygous gyro male (Gy/Y) mice overproduce spermidine and exhibit increased resistance

to oxidative stress but decreased resistance to UV irradiation. Biochem. J. 2000, 352, 381–387.
20. *Ogiso S., Matsumoto T., Nimura Y.*: The role of polyamines in liver regeneration after hepatectomy with ischemic injury. Surg. Today 1997, 27, 833–839.
21. *Ohkawa H., Ohishi N., Yagi K.*: Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. Anal. Biochem. 1979, 95, 351–358.
22. *Ohmori S., Misaizu T., Kitada M., Kitagawa H., Igarashi K., Hirose S., Kanakubo Y.*: Polyamine lowered the hepatic lipid peroxide level in rats. Res. Commun. Chem. Pathol. Pharmacol. 1988, 62, 235–249.
23. *Paridaens R., Uges D.R., Barbet N., Choi L., Seeghers M., van der Graaf W.T., Groen H.J., Dumez H., Buuren I.V., Muskiet F., Capdeville R., Oosterom A.T., de Vries E.G.*: A phase I study of a new polyamine biosynthesis inhibitor, SAM486A, in cancer patients with solid tumours. Br. J. Cancer 2000, 83, 594–601.
24. *Poduslo J.F., Curran G.L.*: Increased permeability of superoxide dismutase at the blood-nerve and blood-brain barriers with retained enzymatic activity after covalent modification with the naturally occurring polyamine, putrescine. J. Neurochem. 1996, 67, 734–741.
25. *Rao J.N., Li L., Bass B.L., Wang J.Y.*: Expression of the TGF-beta receptor gene and sensitivity to growth inhibition following polyamine depletion. Am. J. Physiol. Cell. Physiol. 2000, 279, C1034-C1044.
26. *Ray R.M., Viar M.J., Yuan Q., Johnson L.R.*: Polyamine depletion delays apoptosis of rat intestinal epithelial cells. Am. J. Physiol. Cell Physiol. 2000, 278, C480–C489.
27. *Shin Y., Han S.*: Nitric oxide protects Cu,Zn-superoxide dismutase from hydrogen peroxide-induced inactivation. FEBS Letters 2000, 479, 25–28.
28. *Spencer J.P., Whiteman M., Jenner A., Halliwell B.*: Related nitrite-induced deamination and hypochlorite-induced oxidation of DNA in intact human respiratory tract epithelial cells. Free Radical Biol. Med. 2000, 28, 1039–1050.
29. *Yoon S.J., Koh Y.H., Floyd R.A., Park J.W.*: Copper, zinc superoxide dismutase enhances DNA damage and mutagenicity induced by cysteine/iron. Mutat. Res. 2000, 448, 97–104.
30. *Zavodnik I.B., Lapshina E.A., Rekawiecka K., Zawodnik L.B., Bartosz G., Bryszewska M.*: Membrane effects of nitrite-induced oxidation of human red blood cells. Biochim. Biophys. Acta 1999 1421, 306–316.

Otrzymano: 2001.03.16