Amino Acids (2007) 32: 413–417
DOI 10.1007/s00726-006-0382-2
Printed in The Netherlands

Amino Acids

Protective effect of taurine on respiratory burst activity of polymorphonuclear leukocytes in endotoxemia

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Received June 15, 2006
Accepted July 12, 2006
Published online October 2, 2006;

Summary. The aim of this study was to evaluate the effect of endotoxin on PMN leukocyte respiratory burst activity by measuring G6PD, NADPH oxidase and XO activities in guinea pig. In addition, the possible protective role of taurine against endotoxin-mediated PMN leukocyte function was examined. All experiments were performed with four groups (control, taurine, endotoxemia, taurine plus endotoxin) of ten guinea pigs. After the endotoxin was administrated (4 mg/kg) both G6PD and NADPH oxidase activities were significantly reduced compared with the control group. NADPH oxidase activity returned to the control value and G6PD activity also increased but it did not reach the control value. However when taurine was administrated (300 mg/kg) the activity of NADPH oxidase reached the control value; furthermore, G6PD activity also increased but it could not reach to the control value. When taurine was administrated alone, no effect on these enzymes was observed. Following the endotoxin administration, the activity of XO considerably increased. When taurine was administrated together with endotoxine and alone, this activity decreased compared to control value in both conditions. These results indicate that the $O_2^{\bullet -}$ formation in PMN leukocytes after the endotoxin administration is ensured by the catalysis of XO due to the inhibited NADPH oxidase activity. It was observed that taurine has considerable anti-inflammatory and antioxidant effects. However, conflicting results were obtained when taurine was administrated alone or together with an oxidant agent.

Keywords: Respiratory burst – Polymorphonuclear leukocyte – Taurine – Endotoxemia

Abbreviations: ROS, reactive oxygen species; $O_2^{\bullet -}$, superoxide; G6PD, glucose-6-phosphate dehydrogenase; NADPH, nicotinamide adenine dinucleotide phosphate; NO, nitric oxide; XO, xanthine oxidase; PMN, polymorphonuclear; LPS, lipopolysaccharide; ip, intraperitoneally; iNOS, inducible nitric oxide synthase; $ONOO^-$, peroxynitrite

Introduction

Phagocytes are the first line defence against invading pathogens, killing and disposing of them with impressive efficiency. Stimulated phagocytes, under inflammatory conditions, generate a variety of well characterised reactive oxygen species (ROS). Neutrophils and other phagocytes manufacture superoxide ($O_2^{\bullet -}$) by one electron reduction of oxygen at the expense of NADPH. Most of $O_2^{\bullet -}$ reacts with itself to form H_2O_2. From these agents a large number of highly reactive microbicidal oxidant was formed. These reactive oxidants are generated for the purpose of killing invading microorganisms but they also inflict damage on nearby tissues (Babior, 2000; Evans et al., 1996; Marcinkiewicz et al., 2000).

Glucose-6-phosphate dehydrogenase (GPDH) is an abundant cytosolic enzyme, and catalyzes the reduction of nicotinamide adenine dinucleotide phosphate (NADP) to NADPH. Phagocytic leucocytes use NADPH as a substrate for the NADPH oxidase enzyme. NADPH oxidase, a membrane-bound heme protein of neutrophils, is a primary source of inflammatory cell derived $O_2^{\bullet -}$. In polymorphonuclear (PMN), it was found the correlation among G6PDH activity, $O_2^{\bullet -}$ release and NADPH oxidase activity (Babior, 2000; Batchelor and Zhou, 2004; van Bruggen et al., 2002; Wolach et al., 2004). In addition to ROS, neutrophils can also simultaneously produce NO through the activation of iNOS when these cells are exposed to cytokine or lipopolysaccharide (LPS) (Babior, 2000; Batchelor and Zhou, 2004; Evans et al., 1996; Kristof et al., 1998; Marcinkiewicz et al., 2000; van Bruggen et al., 2002; Wolach et al., 2004).

In previous reported data, it was indicated that NO (nitric oxide) inhibits $O_2^{\bullet -}$ production via direct effects on the membrane components of the NADPH oxidase (Sethi and Dikshit, 2000). Therefore the production of $O_2^{\bullet -}$ and bactericidal activity of neutrophils should be inhibited or

blocked. It raised the question as to the source of $O_2^{\bullet -}$ when phagocytes were exposed to their targets. The answer was provided by Ralf et al. who indicated that expression of xanthine oxidase (XO) was increased after LPS treatment as well as NADPH oxidase (Brandes et al., 1999). Among several mechanisms, XO may be a potential source for the enhanced oxidative stress. Xanthine oxidoreductase can exist in two different forms, xanthine dehydogenase or XO. XDH normally reduces NAD^+ while XO accepts an electron from oxygen (Babior, 2000; Brandes et al., 1999; Wallwork et al., 2003). Thus XO generates $O_2^{\bullet -}$ and H_2O_2. In view of this knowledge, the measurement of XO in neutrophils is seen important for the production of $O_2^{\bullet -}$.

On the other hand, taurine (2-aminoethanesulfonic acid), a sulphur-containing β-amino acid, a semi-essential, antioxidative amino acid is not incorporated into proteins. In mammalian tissues, taurine is the most abundant free amino acid in leukocytes. Previous studies reported that, taurine possess potent antimicrobial properties as it is able to increase neutrophils phagocytic ability and respiratory burst activity (Chorazy et al., 2002; Egan et al., 2001; Kim et al., 1998; Kwasny-Krochin et al., 2002; Marcinkiewicz et al., 1999). After exposure to bacterial LPS, it is known that the respiratory burst activity may be increased. However, the mechanism under lying endotoxin-induced $O_2^{\bullet -}$ generation is not clear.

The aim of this study was to compare NADPH oxidase and XO activity in their ability to generate of the $O_2^{\bullet -}$ anion. It is produced by NADPH oxidase or XO and which is more dominant in the producing of $O_2^{\bullet -}$. Furthermore in this study we evaluated the effect of taurine on endotoxin administrated in a guinea pig model.

Therefore, NADPH oxidase, XO and G6PD activities in PMN leukocytes were measured following taurine, endotoxin and taurine plus endotoxin administration.

Materials and methods

Reagents

NaOH, Na_2CO_3, KCl, $CuSO_4$, KCN, Na_2HPO_4, KH_2PO_4, glucose, HCl, and $MgCl_2$ used for the study were purchased from Merck Chemical Co. (Darmstadt, Germany). Xanthine, NADPH, glucose-6-phosphate, NADP were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

Animals and study protocol

All experiments were performed with 40 adult male Dunkin Hartley guinea pigs weighing 500–600 g. The animals were randomized into four groups. Group I ($n = 10$) served as control group and was given only saline solution. Group II ($n = 10$) served as taurine group and was administered intraperitoneally (ip) in a single dose (300 mg/kg) (Egan et al., 2001). Group III ($n = 10$) served as endotoxaemia group and was administered ip LPS (Esherichia coli LPS serotype 0111:B4, from Sigma, 4 mg/kg) (Duffy et al., 2000). Group IV ($n = 10$) served as taurine plus endotoxin group and was administered consecutively taurine and LPS at the same way. At the end of the 6th hour at which both taurine and endotoxin rise up to blood level, all animals were anesthetized with ketamine (60 mg/kg) and xylazine (10 mg/kg), intramuscularly, and blood samples were collected via intracardiac puncture using heparin as an anticoagulant.

PMN isolation

PMN were isolated from 10-ml heparinized blood sample according to the method as described by Cutts (1970). Briefly, for this experiment, blood was collected in a tube containing 4 ml 6.5% dextran and incubated at 4 °C for 45 min. The supernatant was then gently removed and taken into another tube and 20 ml Hanks' solution was added and centrifuged at 4 °C, 2000 rpm for 5 min. The supernatant was removed and sediment mixed with 15 ml distilled water for 30 sec and then 5 ml 3.5% NaCl was added. The mixture was centrifuged at 4 °C, 2000 rpm for 5 min, thereby removing the erythrocytes. The supernatant was removed and the sediment was washed in 20 ml Hanks' solution. The mixture was centrifuged at 4 °C, 2000 rpm for 5 min. The supernatant was removed and 3 ml 0.16 M KCl was added to the sediment and then sonicated at 50 watt, for 30 sec. The mixture was stored at −80 °C until use.

G6PD activity determination

PMN–G6PD enzyme activity was determined spectrophotometrically by monitoring (at 340 nm) the rate of reduction of $NADP^+$ to NADPH in sonicated PMNs. The enzyme activity is determined by measurement of the rate of increase in NADPH concentration (Kornberg et al., 1995).

NADPH oxidase activity determination

PMN–NADPH oxidase enzyme activity was determined spectrophotometrically by monitoring (at 340 nm) the rate of reduction of NADPH to $NADP^+$ in sonicated PMNs. The enzyme activity is determined by measurement of the rate of decrease in NADPH concentration (Beutler, 1975).

XO activity determination

The PMN suspension was incubated for 40 min at 37 °C and then added to the reaction mixture which contained in final concentrations: xanthine (0.17 mM); phosphate buffer (33 mM, pH 7.5). The reaction was carried out at 37 °C and was stopped at 0 and 20 min by addition of 0.1 ml of 100% (w/v) trichloroacetic acid. The mixture was centrifuged at $10{,}000 \times g$ for 15 min. In the clear supernates the uric acid produced from the substrate, xanthine, was measured by the increase in absorbancy at 293 nm, in a spectrophotometer. Blanks contained the identical reaction mixture without xanthine. The enzyme activity was provided by the difference between the rate in the complete reaction and that in the blank (Prajda and Weber, 1975).

Protein concentration was measured by the Lowry method (Lowry et al., 1951).

Statistical analysis

Results were expressed as mean ± standard deviation. Statistical analyses were performed using a software program (SPSS 11.5 for Windows, Chicago, IL, USA). The nonparametric Mann-Whitney *U*-test was used to analyze the significance of the differences between control and experimental groups. For tests of significance a p value of less than 0.05 was considered to be significant.

Results

The PMN G6PD, NADPH Oxidase and XO activity of the four groups is indicated in Table 1.

Table 1. G6PD, NADPH oxidase, and XO activities in PMN leukocytes of experimental groups

	G6PD (μmol/min/mg protein)	NADPH Oxidase (nmol/h/mg protein)	XO (nmol/min/mg protein)
Control group	0.101 ± 0.045	141.73 ± 46.11	6.18 ± 1.80
Taurine group	0.015 ± 0.007^{a}	53.90 ± 16.65^{a}	5.89 ± 1.96
Endotoxemia group	$0.001 \pm 0.0006^{b,c}$	57.00 ± 35.55^{a}	$13.59 \pm 4.38^{a,c}$
Taurine plus endotoxin group	$0.039 \pm 0.020^{a,d,e}$	$156.70 \pm 62.98^{c,e}$	7.07 ± 1.31^{e}

Results were expressed as mean ± standard deviation; [a] $p < 0.001$, compared to the corresponding value of control group; [b] $p = 0.001$, compared to the corresponding value of control group; [c] $p < 0.001$, compared to the corresponding value of taurine group; [d] $p = 0.004$, compared to the corresponding value of taurine group; [e] $p < 0.001$, compared to the corresponding value of endotoxine group

G6PD Glucose-6-phosphate dehydrogenase; *NADPH* nicotinamide adenine dinucleotide phosphate; *XO* xanthine oxidase; *PMN* polymorphonuclear

G6PD activity

Both taurine and endotoxin administration significantly reduced PMN G6PD activity compared with the control group (for both $p < 0.001$). G6PD activity was elevated in the taurine plus endotoxin group, even though it was significantly lower than the control group ($p < 0.001$). The maximal G6PD activity was seen in the control group.

NADPH oxidase activity

Both taurine and endotoxin administration significantly reduced PMN NADPH oxidase activity compared with the control group ($p < 0.001$ and $p = 0.001$ respectively) and the difference of NADPH oxidase activity between taurine group and endotoxemia group was not significant. NADPH oxidase activity of taurine plus endotoxin group was higher than that of control group but it was not statistically significant ($p > 0.05$). The maximal NADPH oxidase activity was seen in the taurine plus endotoxin group.

XO activity

Endotoxin administration significantly increased XO activity and it was significantly higher than that of control group ($p < 0.001$). Although taurine administration alone reduced XO activity, the XO activity of taurine plus endotoxin group was higher than that of control group, but both of these two differences were not not statistically significant ($p > 0.05$). The maximal XO activity was observed in the endotoxemia group and this activity was significantly higher than the other three groups.

Discussion

Neutrophils stimulated by mediators of endotoxemia undergo a respiratory burst which results in increased production of short-lived oxidant species such as $O_2^{\bullet -}$. The generation of $O_2^{\bullet -}$ by neutrophils is thought to be an essential component of their microbicidal and cytotoxic action. G6PD catalyzes the reduction of NADP to NADPH. Phagocytic leukocytes use NADPH as a substrate for the NADPH enzyme which contributes to the killing of ingested microorganisms (Babior, 2000; Evans et al., 1996; Marcinkiewicz et al., 2000).

Taurine is found in high concentrations in leukocytes particularly PMN and it possesses potent antimicrobial properties as it is able to increase neutrophils phagocytic ability and respiratory burst activity (Chorazy et al., 2002; Egan et al., 2001).

In our study, 4 mg/kg doses of endotoxin were injected into guinea pigs. After 6 h following the endotoxin injection PMN leukocytes were harvested. The functions of leukocytes were investigated because inducible nitric oxide synthase (iNOS) is induced by endotoxin maximally at 6 h after treatment (Cimen et al., 2004). Together with endotoxin, taurine was administrated in order to investigate the effect of taurine on these functions.

In this study after treatment of endotoxin both G6PD and NADPH oxidase activities were significantly reduced compared to the control group but XO activity increased. Our results are consistent with the other published data which suggest that NO generated by iNOS activity induced at 6 h after endotoxin treatment inhibits $O_2^{\bullet -}$ production via direct effect on NADPH oxidase (Sethi and Dikshit, 2000). In PMN it was also reported that there was a correlation between G6PD activity and NADPH oxidase activity. On the other hand Brandes et al. (1999) reported that expressions of XO and NADPH oxidase were increased by LPS in a time-dependent manner. George et al. indicated that activated neutrophils produce 8-nitroxanthine by the MPO-nitrite system and peroxynitrite ($ONOO^-$) and they reported that 8-nitroxanthine

enhances $O_2^{\bullet -}$ production by XO (Yeh et al., 2003). We also found that high level of MPO activity in these neutrophils after treatment with endotoxin (unpublished data).

We used taurine as an antioxidant in our study. However, the effect of taurine was different when administration alone or when taurine plus endotoxin were administrated. Although taurin administration increased the endotoxin-mediated decrease of NADPH oxidase, taurine alone did not have any effect on this enzyme compared to the control value.

Previous studies have shown that taurine possesses potent antimicrobial properties and increases neutrophil phagocytic activity and respiratory burst activity (Chorazy et al., 2002; Egan et al., 2001; Kwasny-Krochin et al., 2002; Marcinkiewicz et al., 1999). Bridget et al. measured the respiratory burst activity with flow cytometry and animals treated with taurine had a more pronounced and significant increase in respiratory burst activity (Egan et al., 2001). However taurine alone had no effect. In our study, taurine had an effect on G6PD in the endotoxin group, but it did not increase the activity to the control value. In the same way, treatment of only taurine could not increase G6PD activity compared to the control group. In our study after endotoxin treatment XO activity increased. Treatment with taurine significantly lessened the XO activity compared to the control group values. Taurine alone also had the same effect on XO activity. Taurine acts as an important immunoregulatory factor that tunes the synthesis of cytokines. Taurine is known to be a cytoprotectant and is the only known endogenous inhibitor of iNOS and NO (Chorazy et al., 2002; Egan et al., 2001; Marcinkiewicz et al., 1999). In the absence of NO, it is possible that NADPH oxidase was not inhibited and the $O_2^{\bullet -}$ anion may have been produced by this enzyme. As a result of this, XO activity may decrease. In vivo and in vitro data indicate that one of the initial events in endotoxemia is neutrophil-mediated endothelial injury by the production of toxic oxygen metabolities via XO activity. Therefore treatment with taurine offers the potential benefits of decreased sequestration of neutrophils and decreased tissue injury without compromising antimicrobial function (Brandes et al., 1999; Egan et al., 2001; Wallwork et al., 2003). Taurine is also able to increase the phagocytic ability of the neutrophils and to increase respiratory activity which further scavenges reactive oxygen derivatives. Taurine acts mostly by preventing oxygen free radical tissue injury (Chorazy et al., 2002; Egan et al., 2001; Marcinkiewicz et al., 1999).

In conclusion, an important concept arising from our experimental study is that taurine may induce NADPH oxidase activity responsible for the respiratory burst but may inhibit XO activity which plays an important role in producing abundant $O_2^{\bullet -}$. However, the effect of taurine was different when administrated alone or with endotoxin.

Acknowledgements

This work was supported by research funds provided by Gazi University in Ankara (Project no: 01/2004/15). The first author kindly thanks Vet. Dr. IDE who works at Gulhane Military Medical Academy and coworkers for their continuing support.

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