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TEMEL İLGÜN

KEZBAN YILDIZ DALGINLI

CANAN GÜLMEZ

ONUR ATAKİŞİ

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Changes in the levels of liver HSP70, plasma nitric oxide, and the antioxidative system in an experimentally induced endotoxemia mouse model and the role of reduced glutathione

Temel İLGÜN, Kezban YILDIZ DALGINLI, Canan GÜLMEZ, Onur ATAKİŞİ*
Department of Chemistry, Division of Biochemistry, Faculty of Sciences and Letters, Kafkas University, Kars, Turkey

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Abstract: Endotoxin molecules in lipopolysaccharides are among most important molecules that initiate a cascade of events in sepsis/endotoxemia. Lipopolysaccharide exposure may result in strong immune responses, disrupt the intracellular oxidant/antioxidant balance, and cause excessive reactive oxygen species generation. The purpose of the study was to examine if reduced glutathione (GSH) has a protective role against lipopolysaccharides. The effects of lipopolysaccharide (LPS) or GSH alone or in combination on the levels of the plasma antioxidant system, NO, and liver HSP70 were investigated. A total of 100 Swiss albino mice were divided into 4 groups as Group I (control), Group II (20 µg/kg LPS), Group III (10 mg/kg GSH), and Group IV (20 µg/kg LPS + 10 mg/kg GSH). Blood and liver samples both pre- and post-LPS and/or GSH injections after 1, 3, and 6 h were collected. Total antioxidant capacity was demonstrated with a reduction in response to lipopolysaccharide. Total oxidant capacity was higher after the injection of lipopolysaccharide alone or in combination with GSH. NO levels were elevated in response to lipopolysaccharide. The liver HSP70 level was determined to be higher in the lipopolysaccharide-treated group. These results indicate that exogenously administered GSH may have regulatory effects on liver HSP70 and plasma NO levels, and GSH treatment might have beneficial effects on antioxidant status by inhibiting the increase of oxidant molecules in endotoxemia-induced mice.

Key words: Antioxidant system, endotoxemia, heat shock protein, nitric oxide, reduced glutathione

1. Introduction

Endotoxin is the most important component of external cell walls of gram-negative bacteria and it mainly consists of lipopolysaccharide (LPS) and other bacterial wall components in various proportions. Endotoxin molecules found in the LPS layer are inactive as long as they stay in the cell membrane. Endotoxin created during rapid cellular growth or cell lysis is the key molecule that initiates the chain of events in sepsis/endotoxemia (Niwa et al., 2000). It has been reported that under conditions where gastrointestinal diseases develop as a result of alcohol use, LPS causes septic shock symptoms via septicemia due to the increased permeability of the digestive tract (Fukui et al., 1991). Clinical symptoms are very significant in endotoxic or septic shock, and deaths are usually caused due to multiple organ failure. In many cases, reactive oxygen species (ROS) contribute to organ failure in sepsis and septic shock as mediator molecules. Septic shock is triggered as a result of secondary products created by ROS, such as nitric oxide (NO), superoxide, and hydrogen peroxide. It has been reported that toxicity and lipid peroxidation could be altered due to complex interactions among nitric acid, oxygen radicals, and antioxidants (Hutcheson et al., 1990; Ma et al., 1995).

Internal and external factors that cause physiological and biochemical imbalances in a living being are called stress factors, and the reactions given to these factors by cells are called cellular stress responses. Animal cells are equipped with defense mechanisms against such stress factors. When cells encounter stressors, they synthesize molecules, so-called heat shock proteins (HSPs), to help defend against them (De Maio, 1999). HSPs are 15–110 kDa molecules that are synthesized in numerous stress conditions, such as rapid temperature or salinity increase, lack of oxygen, lack of light, viral agents, exposure to radiation, and heavy metal toxicities (Henle et al., 1998; Clark and Muchowski, 2000; Moseley, 2000).

HSPs are responsible for the organization of the folding of newly synthesized proteins, protection of nuclear integrity and matrix material, repair of unstructured proteins, separation of damaged proteins, localization of proteins into organelles, intake and outtake of proteins to organelles, and proteolysis of polypeptides having irreversible damage (Schlesinger, 1990). HSP70, initially

* Correspondence: onuratakisi@hotmail.com

identified by Ritossa in the salivary gland cells of fruit flies in 1962, plays roles in protecting cells against heat and oxidative damage by binding to hydrophobic residues of proteins during stress. Accordingly, it prevents possible denaturation and aggregation of proteins (Niwa et al., 2000; Clark and Peck, 2009). HSPs are divided into two groups based upon their cellular functions: perpetually synthesized proteins and stress-induced proteins. Perpetually synthesized proteins bind to yet unfolded polypeptide chains and help transfer these into the target organelles. Stress-induced HSPs play important roles by limiting cellular damage, preventing aggregation of proteins, removing damaged proteins, and inhibiting stress-stimulated necrosis (Heikkila et al., 1982; Sorensen and Loeschcke, 2004).

Free radicals are atoms or molecules that contain one or more unconjugated electrons, which make these molecules quite reactive (Cross et al., 1987; Meister, 1994). These molecules that prevent or delay the oxidation of macromolecules such as proteins, lipids, and carbohydrates in cells are called antioxidants, and this process is called antioxidant defense. Free radicals, although they have a very short life span, can interact with macromolecules and cause significant damage in cellular structures and hence their functions. An antioxidant molecule, reduced glutathione (GSH) is a tripeptide that is found in intercellular media. GSH can protect functional proteins against oxidant agents. It has been reported that GSH in the blood is located mostly in erythrocytes and protects them against oxidative damage (Raftos et al., 2010). By keeping sulfhydryl (-SH) groups of proteins in cells, GSH prevents inactivation of proteins and enzymes and plays a role in the detoxification of compounds and the transition of amino acids through membranes (Meister and Anderson, 1983).

NO is a colorless molecule meditating both physiological and pathologic events in the body. It has a very short life span and could be found in almost all organ systems (Archer, 1993). NO plays roles in the regulation of blood pressure in the veins and interactions between the nerves and defense mechanisms (Burgner et al., 1999). NO is one of the most significant molecules in the defense system of an organism in the case of an infection. Infected cells, tumor cells, and bacteria are blocked by NO, which is secreted by macrophages. NO is synthesized by inducible nitric oxide synthase (iNOS) and displays antimicrobial activity. NO in vitro inhibits the growth of most bacteria and parasites. The antimicrobial effects of NO are not directly caused by NO, but are the result of its reactive metabolites produced as a result of the oxidation of NO (Archer, 1993; Burgner et al., 1999). Some aspects of the relationship between endotoxemia and the oxidant/antioxidant system have been studied previously (Alvarez and Boveris, 2004; Payabvash et al., 2006; Doganyigit et al., 2013; Zhang et al., 2014). However, there are still some points that need to be investigated.

The objective of this study was to investigate the effects of exogenously administered GSH on the level of nitric oxide, total antioxidant/oxidant capacity, and plasma lipopolysaccharide and liver HSP70 levels in an endotoxemia model in mice.

2. Material and methods

2.1. Animals

Swiss albino mice, aged 4 months and weighing 32.96 ± 4.3 g, were used in this study. Before starting the study, approval was obtained from the Kafkas University Animal Experiments Local Ethics Committee (Decision No. KAÜ-HADEK: 2011.46). Animals were kept at constant conditions as follows: temperature (23 ± 2 °C), humidity (50 ± 10%), and light (12-h light/dark cycles). All animals were allowed free access to standard chow (Bayramoğlu, Erzurum, Turkey) and ad libitum freshwater. The same experimental conditions were applied to all groups.

2.2. Experimental design

A total of 100 Swiss albino mice were divided equally into 4 groups: Group I (control-untreated, n = 20), Group II (20 µg/kg LPS, n = 27), Group III (10 mg/kg GSH, n = 26), and Group IV (20 µg/kg LPS + 10 mg/kg GSH, n = 27). To determine the control values (Group I), blood samples and liver tissues were taken from untreated animals under anesthesia. Lipopolysaccharides from *Escherichia coli* 0111:B4 (Sigma-Aldrich: L4391) were used as the LPS source and 20 µg/kg LPS was administered to Group II, 10 mg/kg GSH was administered to Group III, and 20 µg/kg LPS + 10 mg/kg GSH was administered to Group IV via intraperitoneal injection. Blood samples and liver tissues were taken from the groups at 1, 3, and 6 h after injections under anesthesia. Plasma samples were obtained by centrifuging the blood samples for 15 min at 3000 rpm. All the samples were stored at –20 °C until analyses.

2.3. Biochemical analyses

Plasma endotoxin levels were determined by pyrochrome method (Limulus Amebocyte Lysate Pyrochrome, Catalog No. CD060), and HSP70 levels were determined using ELISA (Enzo Life Science, Catalog No. ADI-EKS700B). Plasma total antioxidant capacity (TAC) and total oxidant capacity (TOC) were determined using commercial kits (Rel Assay, Gaziantep, Turkey). Plasma NO levels were determined colorimetrically by the acidic Griess reaction as previously reported (Miranda et al., 2001).

2.4. Statistical analysis

Statistical analysis of the data obtained in the study was conducted with SPSS 20 (licensed to the Kafkas University Information Technologies Department). Mean

and standard error were used in data analyses. Kruskal–Wallis H analysis was conducted to determine the differences between the groups. The Mann–Whitney U test was conducted to determine the source of significant differences among groups. The Friedman test was used to determine the variations in time within the groups. Wilcoxon test analysis was utilized to determine the time of the significant difference in that test. Level of significance was accepted as $P < 0.05$.

3. Results

TOC, TAC, NO, and liver HSP70 levels in all of the groups are shown in the Table. HSP70 values at hours 1, 3, and 6 were significantly different among the groups ($P < 0.05$). Further analyses determined that the control levels (Group I) were significantly higher than those of other groups at hour 1; the values obtained in the GSH-administered group (Group III) at hour 3 were higher than those of the LPS-administered group (Group II); and the values obtained in the LPS-administered group at hour 6 were higher than those of the control and other study groups. It was determined that the endotoxin levels in the LPS-administered groups (both Groups II and IV) were over 1584.9 EU/mL, and the same values in groups that were not administered LPS (Groups I and III) were lower than 0.003 EU/mL. TAC levels in Groups II, III, and IV at the 1st, 3rd, and 6th hours were determined to be significantly lower than those of the control group ($P < 0.05$). TOC levels in response to LPS administration at hour 1 were found to be significantly higher than in the control and GSH-administered groups ($P > 0.05$). Similarly, TOC values in response to both LPS and GSH together at hour 1 were significantly higher than in the control and GSH-administered groups ($P > 0.05$). NO levels at the 3rd and 6th hours in response to LPS were significantly different from those of the other groups ($P < 0.05$); however, there was no significant difference in the measurements taken at hour 1 among all the groups ($P > 0.05$). NO levels obtained in response to LPS at hour 3 were higher than those of the control. GSH and LPS/GSH administrations both resulted in an increase in NO relative to the control group. At the 6th hour after injections, NO levels obtained in the LPS-administered group were significantly higher than those for GSH alone and the combination of LPS/GSH. Furthermore, LPS/GSH administration resulted in higher NO levels than the control treatment and GSH alone.

Analysis of the variations of the parameters demonstrated that HSP70 levels significantly changed only in response to LPS at the 1st, 3rd, and 6th hours. The values of HSP70 recorded at hour 6 were higher than those recorded at other times. It was also determined that TAC levels obtained at the 1st, 3rd, and 6th hours were significantly higher than that of the control. TOC levels determined for LPS/GSH demonstrated a significant difference based on time ($P < 0.05$). The values measured at hour 1 were higher than the measurements conducted at hour 6 and for the control group. NO levels demonstrated a significant difference between the times of measurement for LPS administration and LPS/GSH ($P < 0.05$). The values measured in response to LPS at hour 6 were higher than the control values (hour 0) and the measurements taken at hour 3, and the values at the hour 6 for the LPS/GSH group were higher than the values at hours 0, 1, and 3.

4. Discussion

Infection may result in a full-body inflammatory response that may ultimately lead to incurable hypotension. This condition is referred to as septic shock. It has been reported that the mortality rate due to septic shock in humans ranges between 20% and 80% (Van Amersfoort et al., 2003; Opal, 2007). Patients with sepsis present increased concentrations of markers of oxidative damage and decreased concentrations of plasma antioxidants. In animal models, antioxidant support has proven to be beneficial in reducing the oxidative stress caused by endotoxins in various tissues (Ogilvie et al., 1991; Galley et al., 1996; Cadenas et al., 1998). This study aimed to investigate the effects of GSH on the plasma antioxidant status and LPS and liver HSP70 levels in experimentally endotoxemia-induced mice. Studies recorded that an empirical endotoxemia model could be created based on the differences in the dose and frequency of administrations (Opal, 2007). In the present study, it was shown that the endotoxin levels in blood samples taken at hours 1, 3, and 6 after 20 µg/kg endotoxin intraperitoneal injections were all higher than 1584.9 EU/mL, demonstrating that LPS injected intraperitoneally entered the blood circulation in less than 1 h and stayed in the circulation until the last hour of sampling. It is known that HSPs are a protein family that exists in all cells and is produced as a response to factors such as a temperature increase or decrease, toxicity, and stress (Clark and Muchowski, 2000; Moseley, 2000). In another study, 10 ng/mL and 100 ng/mL LPS did not increase HSP60 and HSP70 levels in cultured rat microvascular pericytes after 4 h when compared to the control group; however, after 18 h, HSP60 and HSP70 levels increased (Burgner et al., 1999). A study by Bellmann et al. (1996) reported that HSP70 exhibited protective properties against NO production in the presence of heat stress. In another study, it was reported that as a result of the increase in HSP in heart and liver tissues, a sudden nitric oxide increase took place as a result of iNOS (Malyshev et al., 1995). In a study conducted in a cell culture, it was determined that the nitric oxide-synthase inhibitor L-NNA decreased the synthesis of HSP70 levels by 2-fold to 3-fold, dependent on the temperature (Malysheva et

Table. HSP70, endotoxin, total antioxidant capacity, total oxidant capacity, and nitric oxide levels in samples.

Parameters	Groups	Before experiment	1st hour	3rd hour	6th hour	Significance
Heat shock protein 70 (ng/mL)/g liver tissue	Group I	53.93 ± 10.07^{a}	53.93 ± 10.07^{x}	53.93 ± 10.07^{x}	53.93 ± 10.07^{x}	NS
	Group II		38.29 ± 6.83^{by}	49.22 ± 5.12^{cy}	85.63 ± 10.90^{dy}	$\chi^2_F = 10.714$, P = 0.013 (d > a,b,c)
	Group III		32.31 ± 8.83^{z}	63.08 ± 11.91^{z}	56.11 ± 9.92^{z}	$\chi^2_F = 2.829$, P = 0.419
	Group IV		41.41 ± 5.26^{w}	56.91 ± 11.05^{w}	40.35 ± 9.76^{w}	$\chi^2_F = 1.950$, P = 0.583
	Significance		$\chi^2_{KW} = 18.31$, P = 0.000 (x > y,z,w)	$\chi^2_{KW} = 21.24$, P = 0.000 (z > y)	$\chi^2_{KW} = 8.78$, P = 0.000 (y > x,z,w)	-
Endotoxin (EU/mL)	Group I	<0.003	<0.003	<0.003	<0.003	NS
	Group II		>1584.9	>1584.9	>1584.9	NS
	Group III		<0.003	<0.003	<0.003	NS
	Group IV		>1584.9	>1584.9	>1584.9	NS
	Significance		-	-	-	-
Total antioxidant capacity (mmol Trolox equiv./L)	Group I	1.52 ± 0.082^{a}	1.52 ± 0.082^{x}	1.52 ± 0.082^{x}	1.52 ± 0.082^{x}	NS
	Group II		0.92 ± 0.012^{by}	0.83 ± 0.011^{cy}	1.08 ± 0.18^{dy}	$\chi^2_F = 10.714$, P = 0.013 (a > b,c,d)
	Group III		$0.90 \pm 0.016c^{bz}$	1.07 ± 0.06^{cz}	1.13 ± 0.17^{dz}	$\chi^2_F = 11.850$, P = 0.008 (a > b,c,d)
	Group IV		$1.06 \pm 0.065d^{bw}$	1.04 ± 0.05^{cw}	1.09 ± 0.17^{dw}	$\chi^2_F = 15.00$, P = 0.002 (a > b,c,d)
	Significance		$\chi^2_{KW} = 18.31$, P = 0.000 (x > y,z,w)	$\chi^2_{KW} = 21.24$, P = 0.000 (x > y,z,w)	$\chi^2_{KW} = 8.78$, P = 0.032 (x > y,z,w)	-
Total oxidant capacity (µmol H_2O_2 equiv./L)	Group I	15.40 ± 1.38^{a}	15.40 ± 1.38^{x}	15.40 ± 1.38	15.40 ± 1.38	NS
	Group II		25.48 ± 4.1^{y}	21.34 ± 2.8	20.38 ± 2.1	$\chi^2_F = 4.543$, P = 0.208
	Group III		16.06 ± 2.1^{z}	21.50 ± 3.8	21.31 ± 5.2	$\chi^2_F = 1.950$, P = 0.583
	Group IV		24.44 ± 2.4^{bw}	21.22 ± 2.2^{c}	16.09 ± 2.3^{d}	$\chi^2_F = 8.850$, P = 0.031 (b > d,a)
	Significance		$\chi^2_{KW} = 10.99$, P = 0.012 (y > x,z,w; w>z,x)	$\chi^2_{KW} = 6.879$, P = 0.076	$\chi^2_{KW} = 7.09$, P = 0.069	-
Nitric oxide (µmol/L)	Group I	13.24 ± 1.91^{a}	13.24 ± 1.91	13.24 ± 1.91^{x}	13.24 ± 1.91^{x}	NS
	Group II		18.22 ± 2.43^{b}	37.34 ± 5.12^{cy}	72.79 ± 6.48^{dy}	$\chi^2_F = 9.00$, P = 0.029 (d > c > a)
	Group III		11.85 ± 1.51	13.39 ± 0.96^{z}	11.94 ± 1.36^{z}	$\chi^2_F = 5.40$, P = 0.145
	Group IV		14.77 ± 2.17^{b}	22.85 ± 4.90^{cw}	40.28 ± 4.14^{dw}	$\chi^2_F = 11.10$, P = 0.011 (d > a,b,c)
	Significance		$\chi^2_{KW} = 5.01$, P = 0.171	$\chi^2_{KW} = 15.27$, P = 0.002 y > x,z; w > x	$\chi^2_{KW} = 22.70$, P = 0.000 y > x,w,z; w > z,x	-

Different superscript letters within the same column (a, b, c) and row (x, y, z) indicate significant differences. χ^2_{KW}: Kruskal–Wallis test, χ^2_F: Friedman test, Group I: control, Group II: 20 µg/kg LPS, Group III: 10 mg/kg GSH, Group IV: 20 µg/kg LPS + 10 mg/kg GSH.

al., 1996). Byrne and Hanson (1998) incubated gastric mucosa cells with S-nitroso-N-acetyl-penicillamine for 8 h and observed increases in HSP72 production directly proportional to concentration. It could be argued that determination of significant increases in HSP70 and similarly in NO levels in liver tissue 6 h after the injection in the LPS-administered group, similar to the findings of the above-mentioned studies, could demonstrate a relationship between HSP70 and NO (Bellmann et al., 1996; Malysheva et al., 1996).

In a study conducted on the effects of physiological stress on oxidative damage, antioxidant/oxidant levels, and HSP70 secretion, stressed rats displayed behavioral changes when compared to the control group, and reduced SOD, GSH-Px, and catalase activities and increases in malondialdehyde (MDS) and HSP70 levels were reported (Li et al., 2011). In general, it has been reported that in circumstances that cause stress formation such as high temperatures, anoxia, oxidizing agents, heavy metals, free oxygen radicals, cancer, chemical substances, pesticides, viral infection of the cell, and infection, heat shock proteins, by clearing irreversibly denatured proteins, could assist in the stabilization of macromolecules (Goldring et al., 2000).

NO is the most important molecule serving the defense system of an organism during the presence of an infection. There is a close relationship between the production of NO, synthesized by NO synthetase stimulated by cytokines during infection, and antimicrobial activity. Experimental studies (Lima-Junior et al., 2013; Bogdan, 2015) demonstrated that cytokines such as TNF-α, IL-1, IL-2, and lipopolysaccharides, by simulating the iNOS enzyme, cause an increase in NO levels. The findings of this study demonstrated increased NO levels both in LPS- and both GSH/LPS-administered groups. These findings show that NO has a significant role in antiinfection defense mechanisms.

In one study in which human umbilical vein endothelia cells were treated with LPS for 24 h at various concentrations, an increase in MDA level and a decrease in GSH-Px activity were determined (Zhao et al., 2012). In another study conducted to investigate the protective effects of GSH in mice with LPS-induced acute lung injury, it was reported that LPS significantly increased the formation of reactive oxygen types, and a decrease in GSH levels, an essential extracellular and intracellular protective antioxidant molecule, was observed (Aggarwal et al., 2012). Nandi et al. (2010) investigated antioxidant enzyme levels and interleukin levels in endotoxemia created by intraperitoneally injecting 5 µg of LPS into mice, and they observed increases in tissue catalase and superoxide dismutase antioxidant enzymes as a result of cytokine stimulation. Jaja-Chimedza et al. (2012) analyzed the effects of LPS on glutathione-S-transferase (GST), glutathione peroxidase (GPx)/glutathione reductase (GR), superoxide dismutase (SOD), and catalase (CAT) enzymes in zebra danio (*Danio rerio*) embryos and reported that GST levels decreased in the LPS-administered group. In a study conducted on endotoxin-induced liver toxification, significant increases in lipid peroxidation products in liver homogenates of rats intraperitoneally injected with 1 mg/kg LPS were observed, while decreases in GSH and SOD levels were recorded (Kaur et al., 2006). Thus, in this study, it could be argued that the increase in TOC and the decrease in TAC could be the result of the increase in lipid peroxidation products.

In conclusion, the present study clearly demonstrates that reduced glutathione balanced liver HSP70 and plasma NO levels and prevented the increase of oxidant molecules in an LPS-induced experimental endotoxemia model; however, it did not have any effects on plasma endotoxin levels. These results suggest that reduced glutathione administration had a protective role against the cellular damaging effects of lipopolysaccharides.

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