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Effects of vitamin A and C and melatonin on 3-nitrotyrosine formation in guinea pig heart under lipopolysaccharide-induced stress

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Aim: Bacterial lipopolysaccharide (LPS) triggers synthesis of nitric oxide (NO) and superoxide ($O_2^{\cdot-}$). $O_2^{\cdot-}$ can react with NO to produce the powerful oxidant peroxynitrite ($ONOO^-$). The presence of 3-nitrotyrosine (3-NT) in tissues is often used as a marker of $ONOO^-$ production. Vitamin A, vitamin C, and melatonin are potent antioxidant molecules. We sought to clarify this issue by examining the effects of vitamin A (15,000 IU/kg/day), vitamin C (600 mg/kg/day), and melatonin (25 mg/kg) on 3-NT formation in heart tissue in a guinea pig model of LPS-induced endotoxemia.

Materials and methods: A total of 75 animals were randomly divided into 5 groups (n = 15 animals for each group). 3-NT levels in the heart tissue were determined by high pressure liquid chromatography with a diode array detector.

Results: In the group given LPS, 3-NT levels were significantly increased compared with the control, vitamin A + LPS-treated, vitamin C + LPS-treated, and melatonin + LPS-treated groups. In the vitamin A + LPS-treated group, vitamin C + LPS-treated group, and melatonin + LPS-treated group, 3-NT levels were similar to those of the control group.

Conclusion: Vitamin A, vitamin C, and melatonin pretreatment significantly prevented 3-NT formation. These agents may offer an advantage in that they could improve the hemodynamics as well as reduce the formation of ONOO-.

Key words: Lipopolysaccharide, peroxynitrite, 3-nitrotyrosine, vitamin A, vitamin C, melatonin

Lipopolisakkaritle strese neden olunmuş guinea pig kalbinde 3-nitrotirozin oluşumu üzerine vitamin A, C ve melatoninin etkileri

Amaç: Bakteriyel lipopolisakkarit (LPS), nitrik oksit (NO) ve süperoksit ($O_2^{\cdot-}$) sentezini tetikler. $O_2^{\cdot-}$güçlü oksidan peroksinitrit ($ONOO^-$)'i üretmek için NO ile reaksiyona girebilir. Dokularda 3-nitrotirozin (3-NT)'in varlığı, $ONOO^-$ üretiminin bir belirteci olarak sıklıkla kullanılır. Vitamin A, vitamin C ve melatonin güçlü antioksidan moleküllerdir. LPS ile uyarılmış endotokseminin bir guinea pig modelinde, kalp dokusunda 3-NT oluşumu üzerine vitamin A (15.000 IU/kg/gün), vitamin C (600 mg/kg/gün) ve melatoninin (25 mg/kg) etkilerini inceleyerek bu konuyu açıklamak istedik.

Yöntem ve gereç: Toplam 75 hayvan rasgele 5 (her bir grup için n = 15 hayvan) gruba ayrıldı. Kalp dokusunda 3-NT düzeyleri, diyot array dedektörlü yüksek basınçlı sıvı kromatografi ile belirlendi.

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Bulgular: Kontrol grubu, vitamin A + LPS-verilen, vitamin C + LPS-verilen ve melatonin + LPS-verilen gruplarla karşılaştırıldığında; LPS verilen gruptaki 3-NT düzeyleri önemli bir şekilde yüksekti. Vitamin A + LPS-verilen grup, vitamin C + LPS-verilen grup ve melatonin + LPS-verilen gruptaki 3-NT düzeyleri, kontrol grubundakilere benzerdi.

Sonuç: Vitamin A, vitamin C ve melatonin ile erken muamele, 3-NT oluşumunu öneli bir şekilde önledi. Bu ajanlar, hem ONOO- oluşumunu azaltabildiklerinden hem de hemodinamikleri düzeltebildiklerinden dolayı bir avantaj sunabilirler.

Anahtar sözcükler: Lipopolisakkarit, peroksinitrit, 3-nitrotirozin, vitamin A, vitamin C, melatonin

Introduction

Bacterial lipopolysaccharide (LPS), the cell wall component of all gram-negative bacteria including *Escherichia coli* (*E. coli*), causes the systemic inflammatory response syndrome and septic shock, which finally results in multiorgan failure in humans and animal models. LPS triggers the synthesis and release of cytokines, nitric oxide (NO), and reactive oxygen species (ROS) such as superoxide ($O_2^{\cdot-}$), especially in the lung, liver, and kidney (1,2).

NO is a free radical species that possess a reactive unpaired electron. It is produced in large amounts during sepsis by a nitric oxide synthase, which is inducible (iNOS) upon stimulation by LPS and/or cytokines (3). Intraperitoneal injection of LPS induces the expression of iNOS protein in many cells and tissues including kidney (4). An imbalance between formation of ROS and antioxidants in vivo is suggested to play a major role in multiple diseases. The generated ROS have been shown to attack biological molecules including DNA, proteins, and lipids, contributing to the development of cardiovascular and other chronic diseases (5).

$O_2^{\cdot-}$ can react with NO to produce the powerful oxidant peroxynitrite ($ONOO^-$) (6). $ONOO^-$ is difficult to measure in vivo due to its short half-life. $ONOO^-$ causes nitration on the aromatic ring of free tyrosine, and tyrosine residues of proteins. Since this is a characteristic reaction of $ONOO^-$, the presence of 3-nitrotyrosine (3-NT) in tissues or cell cultures is often used as a marker of $ONOO^-$ production (7). Protein tyrosine nitration has proven to be an important biomarker for oxidative stress in various animal disease models as well as in patients with cardiovascular and/or neurodegenerative diseases (8). Many studies revealed the presence of 3-NT in human tissues and fluids due to inflammation and infectious diseases (9). It was also reported that $ONOO^-$ induced various oxidative damage in vitro, for example low density lipoprotein oxidation, lipid peroxidation, and DNA strand breakage. Various antioxidants have been reported to have an inhibitory effect on the nitration of tyrosine as well as oxidation by $ONOO^-$ (7).

Vitamin A is a fat-soluble essential micronutrient for humans and animals with a variety of biological actions including morphogenesis, vision, immune function, and reproduction (10,11). It has diverse actions on cellular growth and differentiation that go far beyond its classically defined role in vision. b-carotene is the main source of vitamin A, with antioxidant and free radical scavenging activity (10).

Vitamin C, a water-soluble micronutrient essential for humans, is a powerful antioxidant acting both directly via scavenging of ROS and indirectly through regeneration of other antioxidant systems. It has been proven that oxidative damage to macromolecules in vivo can be diminished by supplementation with large doses of this vitamin (11).

Melatonin was found in hundreds of studies to be an effective free radical scavenger, and an antioxidant that protects the lung and liver against the damage induced by several oxidative agents produced by LPS. Furthermore, melatonin is also a scavenger of $ONOO^-$, and it inhibits the production of NO (12). Few authors have found the agent ineffective (13). Both properties of melatonin may be involved in its protective effect on LPS-induced sickness behavior (14).

The objective of the present study was to evaluate the effects of LPS-induced free radicals in guinea pig heart tissues by measuring 3-NT levels. In addition, the possible protective and/or preventive effects of vitamin A and C and melatonin against LPS-mediated $ONOO^-$ formation were assessed.

Materials and methods

This study was approved by the Institutional Committee of Animal Care and Use in Gülhane Military Medical Academy, and all experiments were performed in accordance with the National Institutes of Health Guidelines for the Care and Handling of Animals.

3-Nitro-L-tyrosine and melatonin were obtained from the Sigma Chemical Co. (St. Louis, MO, USA). Vitamins A and C were obtained from Hoffman La-Roche (İstanbul, Turkey). H_2O_2, sodium acetate (NaOAc), citrate, NaOH, H_3PO_4, methanol, HCl, KH_2PO_4, and K_2HPO_4 were purchased from the Merck Chemical Co. (Germany). All organic solvents were of HPLC gradient grade.

The study was done in guinea pigs weighing 200-400 g. They were divided into 5 groups (n = 15 animals in each group). Group 1 animals were injected with saline (control group). Group 2 animals were injected with *E. coli* intraperitoneally dosed at 12 × 10^9 colony-forming units/kg (LPS-treated group) (15). Group 3 animals received vitamin A (15,000 IU/kg/day) 6 days before LPS treatment (vitamin A + LPS-treated group) (16). Group 4 animals received vitamin C (600 mg/kg/day) 4 days after LPS treatment (vitamin C + LPS-treated group) (17). Group 5 animals received melatonin (25 mg/kg) 20 min before LPS treatment (melatonin + LPS-treated group) (18). Animals in group 1 were sacrificed under ether anesthesia 6 h after saline injection. Animals in group 2, group 3, and group 5 were sacrificed under ether anesthesia 6 h after the *E. coli* injection. Animals in group 4 were sacrificed under ether anesthesia 7 days after the *E. coli* injection. After sacrifice, the hearts were removed and immediately frozen in liquid nitrogen. Then the heart tissues were stored at -70 °C until used.

Measurement of 3-nitrotyrosine

A 0.5 g sample of heart tissue in 1.5 mL of buffer (50 mM potassium phosphate buffer, pH 7.4) was homogenized and hydrolyzed in 6 N HCl at 90-110 °C for 18-24 h. Hydrolyzed samples were centrifuged at 3000 rpm for 10 min, and the supernatants were separated for the measurement of the 3-NT levels. The samples were analyzed on a Hewlett Packard 1050 diode array detector HPLC apparatus (Hewlett Packard Waldbron, Germany). The analytical column was a 5-mm pore size Spherisorb ODS–2 C18 reverse-phase column (4.6 × 50 mm; Alltech, Dearfield, IL, USA). The guard column was a C18 cartridge (Alltech Dearfield, IL, USA). The mobile phase was 50 mmol/L sodium acetate / 50 mmol/L citrate / 8% (v/v) methanol (pH 3.1). HPLC analysis was performed under isocratic conditions at a flow rate of 1 mL/min and the diode array detector was set at 274 nm. 3-NT peaks were identified from their retention time, and confirmed by 'spiking' with added exogenous 3-NT. Concentrations of 3-NT were calculated from a 3-NT standard curve, and expressed as nmol/g-tissue (15,19,20).

Statistical analysis

3-NT levels were undetectable in the groups apart from the LPS-treated one. Therefore, a comparison between the groups was not made. We used SPSS® 13.0 for Windows® for statistical analysis.

Results

3-NT levels were not detectable in control group hearts (Table and Figure). 3-NT was only detectable in heart tissue homogenates in the LPS-treated group (group 2). 3-NT concentrations in our HPLC system were below the detection limit in the other 4 groups. LPS injection caused an increase in 3-NT levels (5.22 ± 0.59 nmol/g-tissue) when compared to the control, vitamin A + LPS-treated (group 3), vitamin C + LPS-treated (group 4), and melatonin + LPS-treated (group 5) groups. 3-NT levels in groups 3, 4, and 5 were similar to those in the control group, i.e. all undetectable.

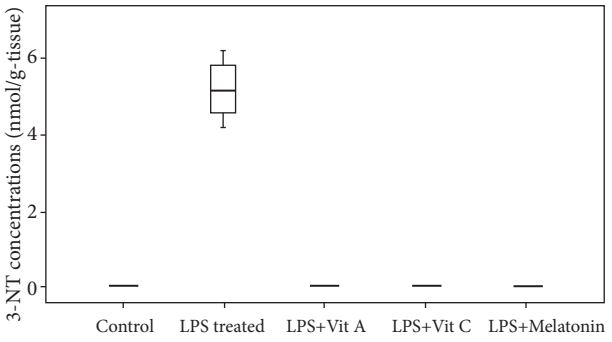

Figure. 3-Nitrotyrosine levels in guinea pig heart homogenates of all groups. LPS: Bacterial lipopolysaccharide; Vit A: Vitamin A; Vit C: Vitamin C.

Table. 3-NT levels in heart homogenates.

	Control Group (n = 15)	LPS-Treated (n = 15)	LPS+Vit A (n = 15)	LPS +Vit C (n = 15)	LPS + Melatonin (n = 15)
3-NT (nmol/g-tissue)	Undetectable	5.22 ± 0.59*	Undetectable[§]	Undetectable[†]	Undetectable[‡]

Numbers represent mean ± SD

* compared with the control group

[§] compared with the LPS-treated group

[†] compared with the LPS-treated group

[‡] compared with the LPS-treated group

LPS: Bacterial lipopolysaccharide; Vit A: Vitamin A; Vit C: Vitamin C

Discussion

Bacterial LPS plays a basic role in sepsis pathogenesis by triggering the release of some mediators and cytokines from various cells. Among these mediators ROS, prostaglandins, and NO have been investigated most intensively. Massive NO production, which seems to contribute to tissue damage in sepsis, results from the induction of iNOS (1). Myocardial iNOS induction has been demonstrated to cause contractile dysfunction in various preparations, including isolated myocytes, isolated perfused working hearts, and in vivo animal preparations. A number of cellular constituents of cardiac muscle, including the endothelium and smooth muscle of the cardiac microvasculature, endocardial endothelium, and cardiac myocytes, are now known to be capable of expressing iNOS in response to LPS and specific cytokines (21). Stimulation of $O_2^{\cdot-}$ and NO generation in response to *E. coli* LPS has been suggested to occur in the lung, liver, and kidney. $O_2^{\cdot-}$ and NO undergo a bi-radical addition reaction to yield $ONOO^-$, which can react with lipids, proteins, and DNA (1). 3-NT formation has also been observed in the erythrocytes, plasma, liver, lung, and spleen of mice and rats treated with LPS (1,15). Although increased NO production from iNOS may decrease vascular resistance, which is beneficial, high levels of NO may also depress myocardial contractility and, through formation of $ONOO^-$, may cause myocardial damage (21). A study demonstrated that $ONOO^-$ is a major contributor to cytokine-induced myocardial dysfunction (22).

In the present study, 6 h and 7 days after *E. coli* administration, we found increased formation of 3-NT in the guinea pig hearts. Vitamin A, vitamin C, and melatonin pretreatment prevented 3-NT formation significantly. The results suggest that LPS increases NO and/or $O_2^{\cdot-}$ in heart tissue, thereby making the tissue more vulnerable to oxidative stress and $ONOO^-$ attacks. These findings confirm that bacterial endotoxin and proinflammatory cytokines stimulate the production of $ONOO^-$ in guinea pig hearts. In addition, this study provides evidence for enhanced generation of $ONOO^-$ and oxidative stress injury in hearts from LPS-injected guinea pigs. In the endotoxemia models, the release of proximal cytokines such as tumor necrosis factor-alpha and interleukin–1 leads to upregulation of iNOS. Kamisaki et al. reported a prolonged increase in plasma nitrotyrosine levels in LPS injected rats, a dose-dependent effect (23). Melatonin has an inhibitory effect on the expression of the iNOS gene and thus limits the NO production by means of iNOS. In this study, melatonin also inhibited 3-NT formation possibly by reducing the synthesis of NO and its hazardous metabolite $ONOO^-$. Khadour et al. have shown a simultaneous increase in both NO and $O_2^{\cdot-}$ generation in the myocardium of hearts from endotoxemic rats (24).

$ONOO^-$ formation has been detected in several models of injury mediated by reactive oxygen species. 3-Nitrotyrosine-protein adducts have been identified in the myocardium following ischemia-reperfusion injury and in patients with myocarditis. Plasma levels

of 3-NT are also elevated in patients with septic shock and in livers from mice treated with LPS (25). León et al. concluded that melatonin acts as a free radical scavenger of ROS and reactive nitrogen species (RNS) (26). Dugo et al. showed that the antioxidant effect of melatonin is correlated with the inhibition of $ONOO^-$ production and poly (ADP-ribose) synthetase (PARS) activation. In vivo treatment with melatonin significantly reduced $ONOO^-$ formation in a dose-dependent manner and prevented the appearance of DNA damage, decrease in mitochondrial respiration, loss of cellular levels of NAD+, and PARS activation (27).

The results presented here show that melatonin protected the hearts against the formation of 3-NT when administrated prior to LPS injection. In fact, melatonin has been shown to reduce $ONOO^-$-induced protein nitration markedly in homogenates of cardiac tissues, and to prevent much of the oxidatively induced tissue damage, following LPS. The hearts were removed 6 h after administration of *E. coli* because data from guinea pig experiments suggest that LPS stimulates NO formation by an effect on iNOS that is maximal at 6 h and then returns to the baseline by 18 to 24 h. The results of the present study, coupled with the recent data from several groups, support the view that melatonin can exert potent protective effects on 3-NT formation by preventing $ONOO^-$ formation.

Bianca et al. observed a delayed loss of endothelium-dependent relaxations, detected 6 h after LPS-induced shock and demonstrated that pretreatment had protected the endothelium from damage in LPS-treated rats, and that study provided evidence for the marked protective effect of melatonin against endothelial dysfunction (12). Crespo et al. demonstrated that melatonin was able to inhibit the expression of the iNOS mRNA levels induced by LPS in lung and liver of rats in vivo in a dose-dependent manner (28). In another study, Cruz et al. showed that the cardioprotection of melatonin was related to a reduction in malondialdehyde and a restoration of the antioxidant status in the myocardium (29). In addition, Sönmez et al. indicated that alleviation of oxidative stress by antioxidant therapy (melatonin and vitamin C) reduces ROS-mediated NO inactivation in heart tissue of chronic alcoholic rats (30).

Vitamins A, E, and C are reported to act as an effective antioxidant of major importance for protection against diseases and degenerative processes caused by oxidative stress (31). Bo et al. have shown that the intake of antioxidant vitamins (vitamins E, C, and A) is negatively associated with hyperglycemia and plasma 3-NT levels. Moreover, in diabetic rats, only combined therapy with insulin and antioxidant vitamins (but not insulin alone) resulted in a reduction in tissue 3-NT (32). In an animal study, Carlson et al. have shown that antioxidant vitamin therapy (vitamins C, A, and E) abrogates myocardial inflammatory cytokine signaling and attenuates sepsis-related contractile dysfunction. On the other hand, they have suggested that antioxidant vitamin therapy may be a potential approach to treat injury and disease states characterized by myocardial dysfunction (33). Vitamin C has been shown to stimulate the immune system by enhancing T-lymphocyte proliferation in response to infection, leading to increased cytokine production and synthesis of immunoglobulins. Vitamin C may prevent dysregulation of the immune-inflammatory response by its antioxidant properties and vitamin C selectively influences intracytoplasmic cytokine production in monocytes and lymphocytes in vitro (34). Ashton et al. have reported that, following supplementation with ascorbic acid, levels of nitrite and hence NO are decreased in the plasma, suggesting scavenging of $NO/ONOO^-$ by ascorbic acid (35).

Antioxidants, such as vitamins A and C and melatonin, probably play important acute and chronic roles in reducing or eliminating the oxidant damage caused by $ONOO^-$. These may protect tissues against free radical attack by scavenging toxic free radicals formed in heart tissue during LPS, which can impair the antioxidant defense system, and reduce the antioxidant defense potential in the heart tissue. Antioxidant treatments may protect the heart tissues from the toxic effects of LPS. Our results showed that vitamins A and C and melatonin given concomitantly with LPS could protect the heart tissue from free radical injury.

In summary, 6 h following LPS administration, the guinea pig heart showed signs of oxidant stress that were ameliorated by vitamins A and C and melatonin. The presence of 3-NT in the heart tissues indicated

the generation of both NO and $O_2^{\cdot-}$ and the formation of the strong oxidant $ONOO^-$. Production of NO and $O_2^{\cdot-}$ results in generation of RNS and ROS. LPS could cause a heightened environment for oxidant stress that overcomes cellular defense mechanisms, leaving the heart susceptible to oxidant injury. RNS should be considered potential targets for therapeutic intervention. Vitamins A and C and melatonin may offer an advantage in that they could improve hemodynamics as well as reduce the formation of $ONOO^-$.

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