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Abstract
The aim of the work was to study the dealyed effect of lipopolysaccharide (LPS) administration on endothelial function of the aorta of rats with genetic hypertension (SHR). Further, the possibility to ameliorate LPS-induced changes by omega-3 fatty acids (PUFA) was tested. Rats received bolus of 1mg/kg LPS i.p.; PUFA were administered in the dose of 30 mg/kg daily for 10 days p.o.. Ten days after LPS, the body weight gain of LPS rats was statistically lower compared to controls. PUFA administration to LPS rats had no effect on this paramater. The TBARS and NAGA concentrations in plasma were significantly increased in the LPS group and PUFA administration returned them to control values. In functional studies, phenylephrine (PE, 1 μmol/l) evoked contraction of aortas which was not statistically different among experimental groups. However, endothelium-dependent relaxation was depressed in the LPS group and PUFA slightly recovered it to control values. In conclusion, oxidative stress seems to be responsible for aortic endothelial dysfunction detected 10 days after administration of LPS to rats. PUFA slightly improved the function of the endothelium, probably thanks to their antioxidant properties. Prolonged administration of higher doses of PUFA should defend the vascular endothelium against detrimental effect of bacterial inflammation.

Keywords: lipopolysaccharide; omega-3 fatty acids; spontaneously hypertensive rats; aorta

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Consequences of lipopolysaccharide and n-3 polyunsaturated fatty acid administration on aortic function of spontaneously hypertensive rats

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Short title: Effect of lipopolysaccharide in SHR rats

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Abstract

The aim of the work was to study the dealyed effect of lipopolysaccharide (LPS) administration on endothelial function of the aorta of rats with genetic hypertension (SHR). Further, the possibility to ameliorate LPS-induced changes by n-3 polyunsaturated fatty acids (n-3 PUFA) was tested. Rats received a bolus of 1mg/kg LPS i.p.; n-3 PUFA were administered in the dose of 30 mg/kg daily for 10 days p.o.. Ten days after LPS, the body weight gain of LPS rats was statistically lower compared to controls ($p<0.05$). n-3 PUFA administration to LPS rats had no effect on this paramater. The TBARS and NAGA concentrations in plasma were significantly increased in the LPS group ($p<0.05$) and n-3 PUFA administration returned them to control values. In functional studies, phenylephrine (PE, 1 μmol/l) evoked contraction of aortas which was not statistically different among experimental groups. However, endothelium-dependent relaxation was depressed in the LPS group ($p<0.05$) and n-3 PUFA slightly recovered it to control values. In conclusion, oxidative stress seems to be responsible for aortic endothelial dysfunction detected 10 days after administration of LPS to rats. n-3 PUFA slightly improved the function of the endothelium injured by LPS, probably thanks to their antioxidant properties. Prolonged administration of higher doses of n-3 PUFA should defend the vascular endothelium against detrimental effect of bacterial inflammation.

Keywords: lipopolysaccharide, omega-3 fatty acids, spontaneously hypertensive rats, aorta

Introduction

Lipopolysaccharide (LPS) or endotoxin is the glycolipid present in the outer membrane of gram-negative bacteria. It generally consists of a hydrophobic lipid A domain, an oligosaccharide core, and the outermost O-antigen polysaccharide (Raetz and Whitfield 2002). Lipid A is a di-glucosamine-based lipid that serves as a hydrophobic anchor of LPS to the microbial membrane and is the single region of LPS that is recognized by the innate immune system. Slightly elevated levels of LPS persist in humans with chronic diseases and lifestyles that involve chronic smoking and drinking (Morris et al. 2015).

Cellular responses to LPS depend on its dose - high doses cause acute, resolving inflammation, while lower doses are associated with low-grade and chronic non-resolving inflammation. Elevated circulating endotoxin may program host leukocytes into a low-grade "memory" state and contribute to the pathogenesis of diverse diseases including atherosclerosis, diabetes, Parkinson's disease, etc. (Manco et al. 2010; Mehta et al. 2010; Wiesner et al. 2010; Morris et al. 2015).

Recent evidence indicates that the endothelium does not play a passive role in systemic inflammatory states. Endothelial cells produce an abundance of inflammatory mediators and elements of the immune and coagulation systems in the host response to inflammatory stimulation. The barrier function of the endothelium is impaired by endotoxins and is likely to contribute to adverse outcomes. A number of authors have recently developed a concept of the aberrant and dysfunctional endothelial barrier as the central pathophysiological process in LPS-induced inflammation and septic shock (Schouten et al. 2008; Opal and van der Poll 2015).

Endothelial dysfunction is now well established as a pivotal early event in the development of major cardiovascular diseases including hypertension, atherosclerosis and diabetes. Pre-clinical studies have indicated that polyphenol-rich food and food-derived products, e.g. grape-derived products and omega-3 fatty acids, can trigger pathways in endothelial cells promoting an increased formation of NO and endothelium-dependent hyperpolarization. Moreover, intake of such food-derived products has been associated with the prevention and/or the improvement of an established endothelial dysfunction in several experimental models of cardiovascular diseases and in humans with cardiovascular diseases (Auger et al. 2016).

The lipid A component of LPS in picomolar concentrations is sufficient to cause endothelial cell injury by promoting the expression of tissue factor and proinflammatory cytokines like TNF-α and IL1β (Miller et al. 2005), leading to apoptosis of endothelial cells (Bannerman et al. 2002). Different bacterial infections accompany us from birth to old age. Presumably, each inflammation might affect the function of the endothelial membrane and consequently lead to injury of vessels and organs they supply. Regular consumption of compounds with protective membrane effects could be beneficial for normal function of vessels.

Polyunsaturated fatty acids are natural constituents of the diet, with a wide spectrum of physiological effects (Komatsu et al. 2003, Calder 2012). Their anti-inflammatory properties were documented experimentally and confirmed by clinical trials. In cultured macrophages polyunsaturated fatty acids decreased the expression of 10 genes related to inflammation (Allam-Ndoul et al. 2016). Clinical trials on fish oil in patients with rheumatoid arthritis (Kremer et al. 1985) showed a significant anti-inflammatory activity of the combination of eicosapentaenoic (EPA) and docosahexaenoic acid (DHA). Various studies confirmed that chronic consumption of fish oil or omega-3 polyunsaturated fatty acids (n-3 PUFA) reduced atherosclerosis (Shim et al. 2016) and ameliorated heart failure (Chrysohoou et al. 2016). n-3

PUFA protect the function and integrity of the endothelium against injury, preventing structural remodeling of the vascular wall. Our previous studies showed n-3 PUFA diet-induced modulation of Cx43 expression in the aorta and heart of hypertriglyceridemic (HTG) and hypertensive rats (Mitasikova et al. 2008, Dlugosova et al. 2009a,b), as well as modulation of endothelial Cx40 expression in the aorta of Wistar rats injected with LPS (Frimmel et al. 2014), supporting the involvement of intercellular communication in protective effects of n-3 PUFA.

In essential hypertension, in animals as well as in humans, chronic vascular and immune dysfunctions are closely associated. In spontaneously hypertensive rats (SHR), various vascular alterations have been reported, e.g. increased activity of the sympathetic system, endothelial dysfunction, arterial compliance decrease, and medial hypertrophy (Folkow 1982, Chobanian 1990). SHR also exhibit immune abnormalities with defective leukocyte–endothelial cell interactions, depressed T lymphocyte functions, decreased delayed-type hypersensitivity (Dzielak 1990, 1992), and chronic inflammatory process in the cardiovascular system (Hinglais et al. 1994). All these observations suggest direct interaction of the cardiovascular and immune systems in SHR. Thus, the response of the rat organism to LPS administration can be influenced by hypertension.

The aim of the work was to study the effect of LPS on endothelial function of the aorta of rats with genetic hypertension (SHR) 10 days after LPS administration. Further, the possibility to ameliorate LPS induced changes by n-3 PUFA was tested.

Materials and Methods

The investigation conformed to the Guide for the Care and Use of Laboratory Animals. The experiments were approved by the State Veterinary and Food Administration of the Slovak Republic.

Experiments were done on 3-month-old male SHR (Breeding station Dobrá Voda, Slovakia), weighing 220.4±5.4 g. They were housed in a room with air temperature of 22-24 °C, 45-60% humidity and regular light control - 12 hours light and 12 hours dark. They were given standard rodent chow and water *ad libitum*. All procedures were done at the same time during the light phase of the L/D cycle. The acclimatization period lasted 10 days.

The rats were randomly assigned to three experimental groups (each of 8 animals): C - control without any treatment; LPS – rats received bolus of 1mg/kg LPS i.p.; LPS + n-3 PUFA – LPS rats treated with n-3 PUFA in the dose of 30 mg/kg daily for 10 days p.o. by gavage. The chosen dose was based on our previous studies (Dlugosova et al. 2009b). Administration of n-3 PUFA (57 % eicosapentaenoic acid and 43 % docosahexaenoic acid - commercial nutritional supplement MaxiCor, SVUS Pharma, Czech Republic) was started on the same day as that of LPS. Treatment of healthy animals with n-3 PUFA had no effect on the parameters measured and are thus not presented in this work. LPS (*Escherichia coli* serotype 055:B5, Sigma Chemical, Germany) was dissolved in sterile 0.9 % NaCl solution. The rats from the control group were injected with the same volume of sterile 0.9 % NaCl solution. The animals were weighed at the beginning and end of the experiment, systolic blood pressure was measured at the same period by non-invasive plethysmographic method on the rat tails. After 10 days of the experiment, the rats were anesthetized with thiopental (50 mg/kg i.p.) and killed by heart excision. Blood was used for evaluation of markers of oxidative stress and the thoracic aorta was removed. Markers of oxidative stress – concentrations of thiobarbituric acid reactive substances (TBARS) - were determined in plasma according to Esterbauer (1993). The plasma specific activity of lysosomal N-acetyl-β-

D-glucosaminidase (NAGA), a marker of cellular injury, was assayed according to standard methods as described previously in Navarova and Nosalova (1994).

Isolated rat aorta

The thoracic aorta was excised and transferred into oxygenated physiological salt solution (PSS). The arteries were cleaned of adherent tissue and cut into rings, approx. 2-3 mm long. Special care was taken not to damage the endothelium. The rings were mounted between two hooks in water-jacketed (37°C) chambers containing PSS bubbled with a mixture of 95% O_2 and 5% CO_2 at pH 7.4. The composition of PSS was (in mmol/l): NaCl (118.0), KCl (4.7), KH_2PO_4 (1.2), $MgSO_4$ (1.2), $CaCl_2$ (2.5), $NaHCO_3$ (25.0), and glucose (11.0). The preparations were connected to an isometric force transducer (Experimetria Hungary), stretched passively to 10 mN and equilibrated for 60 minutes.

Experimental protocol: After the equilibration period, contraction was induced by submaximal concentration of phenylephrine (PE, 10^{-6} mol/l). At the plateau of the contraction, the effect of acetylcholine in the cumulative concentrations of $10^{-8} – 10^{-5}$ mol/l was tested as a measure of endothelial function. After washing with PSS and reaching the initial tension value, concentration-response curves of sodium nitroprusside (10^{-10} - 10^{-7} mol/l) were performed in PE-precontracted preparations.

Statistical analysis

Data are expressed as mean ± SEM. The mechanical responses are expressed as percentages of the PE-induced contraction. Statistical analysis was performed by using ANOVA with Bonferroni posttest. Statistical significance was indicated at $P < 0.05$. The pD_2 values (- log of concentration inducing 50% of maximal response) were calculated by Graph Pad.

Results

Ten days after a non-lethal dose of LPS (1 mg/kg b.w.), we evaluated the physiological state of the rats. As seen in Fig.1, body weight gain of LPS rats was statistically lower compared to controls. n-3 PUFA administration to LPS rats had no effect on this paramater. Blood pressure changes were not found among the experimental groups. The final systolic blood pressure of each group did not increase significantly in comparison to starting values (Fig.2). The TBARS concentration in plasma was significantly ($p<0.05$) increased in the LPS group (4.06 ± 0.36 μg/mg prot.) and n-3 PUFA administration returned it to the control values (LPS+ n-3 PUFA - 3.49 ± 0.18 μg/mg prot , C - 3.18 ± 0.07 μg/mg prot). Similarly, plasma specific NAGA activity was augmented in the LPS group (from 0.14 ± 0.01 to 0.18 ± 0.02 μg 4-NP/min/mg prot., $P<0.05$) and ameliorated in the LPS + n-3 PUFA group (0.13 ± 0.02 μg 4-NP/min/mg prot.) (Fig.3).

The LPS dose used increases levels of CRP which justify the presence of inflammation (Frimmel et al. 2014).

In functional studies, phenylephrine in the concentration of 1 μmol/l evoked contraction which was not statistically different among the experimental groups (not shown). In the control group, acetylcholine induced endothelium-dependent relaxation with the pD_2 value of 7.78 ± 0.11 and the maximal relaxation of 25.61 ± 4.78% of phenylephrine-induced contraction. However, responses of LPS aortas were attenuated (pD_2 7.39 ± 0.25, maximal response 49.33 ± 8.43% of phenylephrine-induced contraction). n-3 PUFA administered to LPS animals slightly shifted endothelium-dependent relaxation to that of controls - pD_2 value 7.72±0.20, maximal relaxation 41.97±7.31 (Fig.4). No differences between groups were found in the response to sodium nitroprusside (not shown).

Discussion

Single LPS administration caused loss of body weight in SHR rats. Likewise, reduced body weight gain was found in hHTG rats using the same LPS experimental model (Frimmel et al. 2016). Our data correspond with the study of Valles et al. (2000) who demonstrated that even a single exposure to stressors, including LPS, can cause long-lasting reduced food intake and affect consequently the body weight, which is mediated by different metabolic or neural mechanisms (Liu et al. 2016). On the other hand, we did not find changes in systolic blood pressure after LPS administration to SHR. We speculate that this might be due to dose- and route-dependent application of LPS, duration of its influence, as well as higher resistance of SHR to LPS (Bernard et al. 1998).

It is known that administration of LPS or cytokines (tumor necrosis factor or interleukin-1) to animals produces a shock-like syndrome, characterized by low blood pressure and hyporeactivity to vasoconstrictor agents (Fink and Heard 1990, Bone 1991). This response is rapid and it was confirmed also in experiment by Vo et al. (2005). These authors showed in Wistar rats that accute application of LPS to precontracted aortic rings induced endothelium-dependent relaxation which reached maximum in up to 5 hours. Moreover, isolated blood vessels of Wistar rats exposed to LPS or removed from LPS-treated animals had diminished responses to vasoconstrictor agents (Joly et al. 1994, Wu et al. 1995). Effects of LPS on the vascular system, however, seem to be dose-, and time-dependent. In our experiments, we administered a nonlethal dose (bolus of 1mg/kg i.p.) of LPS to SHR rats and observed its effect after 10 days. As expected, the results are impacted by this protocol. Contractions induced by phenylephrine were not influenced probably as a consequence of subsided accute response. The hypotensive effect induced by the toxin seems to be dependent also on the rat strain. The spontaneously hypertensive rat should be more resistant to the hypotensive effect of toxins. However, controversial results were obtained with this model. While Bernard et al.

(1998) found that SHR had a greater ability to resist endotoxin shock than normotensive controls, on the other hand, Yen et al. (1997) observed a shorter survival time in SHR after LPS injection in comparison with normotensive rats.

Whereas LPS-induced vascular hyporesponsiveness to constrictor agents may be due to excessive generation of NO by inducible NO-synthase2 (iNOS) (Vo et al. 2005), the mechanism underlying LPS-induced endothelial dysfunction is still not clear and seems to be dependent also on animal species and vessels used. The endothelium-dependent vasodilatation of the thoracic aorta of Wistar rats was found to be significantly inhibited six hours after 10 mg/kg LPS administration (Uğurel et al. 2016). The endothelial injury was accompanied by decreased eNOS and phospho-eNOS expression. Thus changes of the ratio in eNOS/iNOS production seem to be accountable for vascular response in the acute phase of LPS administration. The most important result of our experiments is that even 10 days after LPS administration changes in endothelial function were demonstrated by reduced endothelium-dependent relaxation. At the same time, we also observed enhanced activity of the lysosomal enzyme NAGA in the LPS group compared to controls. Increased plasma and organ activity of the lysosmal enzyme NAGA indicates cell injury ongoing in the organism during pathological processes. This was found in different pathological experimental models (Nosáľová et al. 2010) as well as in patients with hypertension (Lisowska-Myjak et al. 2011) and with type 1 diabetes mellitus (Mandic and Filipovic 1998). Therefore, augmented NAGA activity in our study indicates cell injury remaining 10 days after LPS administration. Moreover, increased plasma levels of TBARS in animals injected with LPS suggest the presence of oxidative stress in these rats. Oxidative stress is involved in atherogenesis, which is initiated by endothelial injury in cases with cardiovascular risk factors, including diabetes mellitus, hypertension, cigarette smoking, dyslipidemia, obesity, and metabolic syndrome (Husain et al. 2015; Salmanoglu et al. 2016). Participation of oxidative stress in induction of

endothelial dysfunction was confirmed by the beneficial action of antioxidants which were able to suppress the detrimental effect of reactive oxygen species (Sotníková et al. 2008, 2011). Results of Requintina and Oxengruk (2003) indicated that the effect of LPS on lipid peroxidation is dose-, time-, and species dependent. Using the identical experimental model, we recently demonstrated in Wistar rats LPS-induced impairment of endothelium-dependent relaxation of the aorta (Frimmel et al. 2014) accompanied with increased values of NAGA activity and levels of TBARS. On the other hand, Wu et al. (1995) observed significantly impaired acetylcholine-induced relaxation in thoracic aortic rings obtained from WKY rats treated with LPS, but not in those from SHR. As these authors studied effects of 5 mg/kg i.v. LPS during 5 hours, we suppose that this discrepancy may be explained by different experimental protocols used.

In order to confirm endothelial damage, we studied the effect of sodium nitroprusside on the aorta to identify the effect of LPS on endothelium-independent relaxation. No differences among experimental groups were found. Our previous studies, using the same experimental LPS model, also demonstrated that LPS did not affect endothelium-independent relaxation of the aorta in Wistar and hHTG rats (Frimmel et al. 2016). The data indicate changes in endothelial function 10 days after LPS injection.

The importance of n-3 PUFA for the cardiovascular system has come under the spotlight during the last decades. Data from clinical (Colussi et al. 2016) and experimental studies (Mitasikova et al. 2008) support the hypothesis that consumption of n-3 PUFA lowers the risk of cardiovascular diseases. The n-3 index - a percentage of eicosapentaenoic acid {EPA) + docosahexaenoic acid (DHA) of total fatty acids in red blood cells (von Schacky and Harris 2007) - may be a marker of increased cardiovascular risk. Our results showed that n-3 PUFA (30 mg/kg b.w.) administered for 10 days were able to suppress oxidative stress and tissue injury in the LPS group of rats. The influence of n-3 PUFA on the endothelium was not so

evident, although endothelium-dependent relaxation, injured by LPS, was slightly improved. Shim et al. (2016) evoked endothelial injury in the atherosclerosis-induced erectile dysfunction rat model. They found that omega-3 fatty acids in high doses and administered over 4 weeks were able to significantly improve intracavernosal pressure and had a beneficial role against pathophysiological consequences, such as fibrosis or hypoxic damage, in this experimental model. Further, supplementation of 4g/day omega-3 fatty acids to healthy volunteers for 4 weeks significantly decreased postprandial triglyceride elevation and postprandial endothelial dysfunction (Miyoshi et al. 2014). We thus suggest that n-3 PUFA could have a protective effect on endothelial dysfunction induced by LPS when administered over a longer time in higher doses.

In conclusion, oxidative stress seems to be responsible for aortic endothelial dysfunction detected 10 days after administration of LPS to rats. In the dose of 30 mg/kg daily, n-3 PUFA slightly improved the function of the endothelium injured by LPS, probably thanks to their antioxidant properties. Prolonged administration of higher doses of n-3 PUFA should defend the vascular endothelium against detrimental effect of bacterial inflammation.

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Figure legends

Figure 1. Body weight gain in control animals (C), animals which received LPS (LPS), and LPS treated with n-3 PUFA (LPS + n-3 PUFA). Data are means ± S.E.M. from 8 animals. *P<0.05 vs C.

Figure 2. Systolic blood pressure at the beginning (full columns) and the end (laminated columns) of the experiment. Control animals (C), animals which received LPS (LPS) and LPS treated with n-3 PUFA (LPS + n-3 PUFA). Data are means ± S.E.M. from 8 animals.

Figure 3. Plasma TBARS and specific NAGA acitvity (in µg of 4-nitrophenyl NAGA) in control animals (C), animals which received LPS (LPS) and LPS treated with n-3 PUFA (LPS + n-3 PUFA). Data are means ± S.E.M. from 8 animals. *P<0.05 vs C.

Figure 4. Endothelium-dependent relaxation of the aorta of control animals (C), animals which received LPS (LPS) and LPS treated with n-3 PUFA (LPS + n-3 PUFA). Data are means ± S.E.M. from 8 animals. *P<0.05 vs C.

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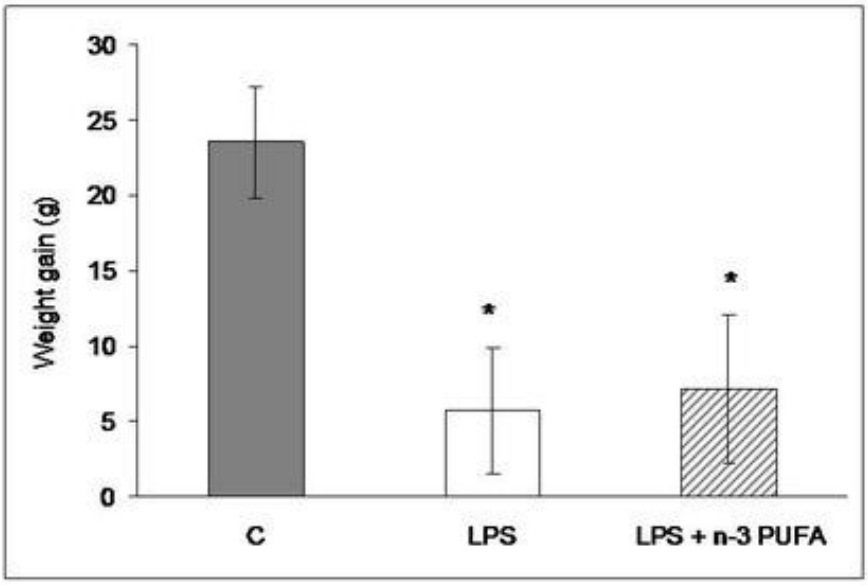

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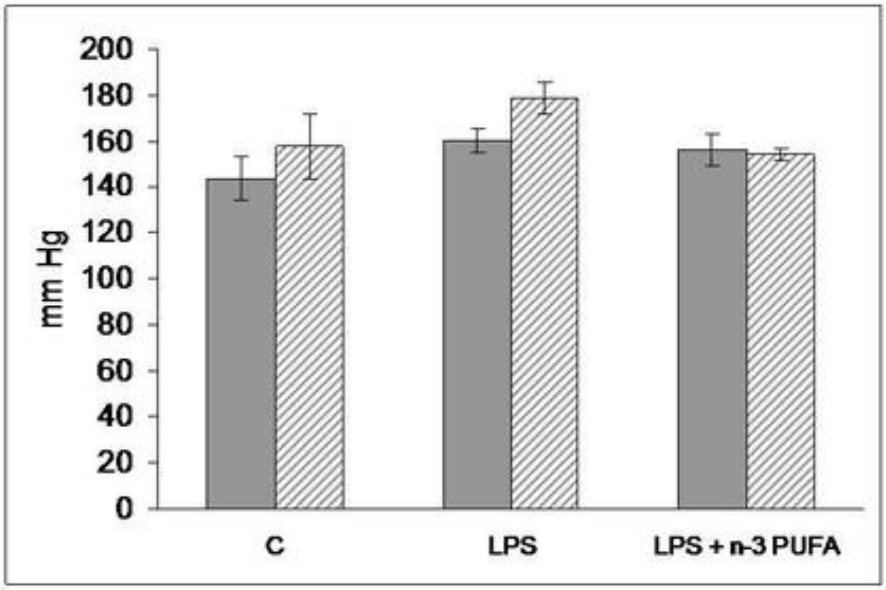

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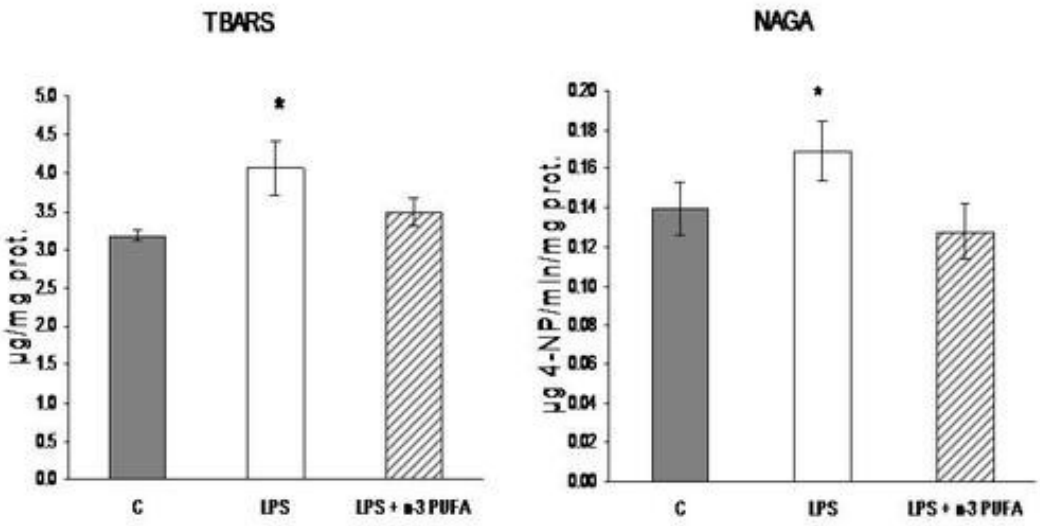

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