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ESCHERICHIA COLI LIPOPOLYSACCHARIDE ADMINISTRATION ALTERS ANTIOXIDANT PROFILE DURING HYPERCHOLESTEROLEMIA

Kallol Dutta and Biswadev Bishayi

Department of Physiology, University College of Science & Technology, University of Calcutta, 92, APC Road, Kolkata 700009, West Bengal, India.

ABSTRACT

Pathogens, especially Gram-negative bacteria or bacterial endotoxin, along with other classical factors, may be involved in inflammatory response within the aortic endothelium during the progression of cardiovascular disease. Studies have shown that bacterial endotoxin activates various inflammatory processes in the body. Our study aims to establish a correlation between endotoxemia and vascular expression of antioxidant enzymes. Swiss albino mice (4 weeks old) were fed a high fat diet for 24 weeks and then were administered Escherichia coli endotoxin intraperitonealy, for 4 weeks. Tissue antioxidant enzymes, serum levels of IL-6 and TNF alpha were measured from the mice. We report that i.p. administration of endotoxin to hyperlipidemic mice resulted in elevation of superoxide dismutase and catalase enzymes, which was paralleled by a systemic reduction of serum cholesterol and LDL expression. Myeloperoxidase levels were also found to be elevated in aortic tissue, while an increase was also observed in the serum cytokine levels.

KEY WORDS

Endotoxin, Hyperlipidemia, Aortic inflammation, Antioxidant enzymes.

INTRODUCTION

Bacterial lipopolysaccharides (LPS; endotoxin) are potent inflammatory agents that have many physiologic and biochemical functions *in vivo*, including increased circulating acute phase proteins (1). Injection of endotoxins into rats resulted in hyperlipidemia (2). Pro-atherogenic effects of endotoxin infusion were observed in cholesterol-fed piglets and rabbits (3, 4). Weekly injections of rabbits with endotoxins significantly elevated atherosclerosis as evidenced by increased aortic lesion area, although no effect of endotoxins was observed on the serum triglycerides or serum low-density lipoprotein (LDL) cholesterol levels (5). Intravenously administered LPS strongly increases the serum level of apolipoprotein E (ApoE) (6). All lipoproteins can bind endotoxins and thereby reduce the toxic properties of LPS.

Address for Correspondence :

Dr. Biswadev Bishayi
Immunology Laboratory, Department of Physiology,
University of Calcutta,
92 APC Road, Kolkata 700009, West Bengal
Phone: +91-33-23508386 Extn: 225.
E-mail: biswa_dev2@yahoo.com

Reactive molecules such as free radicals and lipid peroxides, which can be generated by cells of the arterial wall, may contribute to the formation and progression of aortic atherosclerosis (7). Studies demonstrated the presence of modified LDL *in vivo* and the ability of reactive oxygen species (ROS) added to LDL *in vitro* to convert lipoproteins to a potentially pro-atherogenic form (8). As predicted by the oxidative hypothesis of atherosclerosis, treatments that inhibit the oxidative modification of LDL should prevent atherogenesis (9). Since anti-oxidant enzymes play an important role in controlling lipid peroxidation, an increase in the activities of these enzymes could delay the progression of atherosclerosis (10). An imbalance in the generation of oxidants and anti-oxidants seem to have a vital role in the pathophysiology of atherosclerosis. The expression of the extracellular form of superoxide dismutase (SOD) increases with the severity of atherosclerosis and is associated with an enhancement of SOD activity (11). Because it is now postulated that each of these reactive species activates specific cell-signaling pathways, the importance of this enzyme in regulating cellular responses at sites of inflammation is evident. Emerging evidence indicates that ROS are important risk factors in the pathogenesis of many diseases if the anti-oxidant system is impaired. Because increased circulating lipids and lipoproteins

may give rise to atherosclerotic like syndromes in animals and humans, it is possible that LPS induced modulation of free radical generation (oxidants) is a critical event in the pathogenesis of arterial wall disease. When compared with the considerable literature describing the effects of bacterial LPS on cardiovascular disease, relatively little information is available about any association between LPS and ROS generation or between LPS and anti-oxidant status during hypercholesterolemia. Hypercholesterolemia has been associated with enhanced oxidative stress related to increased lipid peroxidation. Therefore, the inhibition of oxidative stress under hypercholesterolemic conditions is considered to be an important therapeutic approach (12). Taken together, data from numerous studies underscore the notion that modulation of oxidant and anti-oxidant enzymes leading to oxidative stress plays an important role in the pathogenesis of atherosclerotic disease (13). Recently, low levels of Glutathione peroxidase (GPx) activity and elevated levels of myeloperoxidase (MPO) were shown to be independent risk factors for cardiovascular events in patients with coronary artery disease (CAD) and patients presenting with chest pain (14, 15). Maintenance of redox balance in the cardiovascular system is of paramount importance as un-compensated oxidant stress contributes to endothelial dysfunction and vascular disease (16). Studies have also demonstrated that mice over-expressing or under-expressing intracellular anti-oxidant enzymes are potentially valuable models for studying the role of oxidative stress in atherosclerosis (17). Thus when anti-oxidant, free radical scavenging systems are overwhelmed, pathologic conditions may result (18). It can be hypothesized that the anti-oxidant enzyme in aorta or liver may be up-regulated by administration of bacterial LPS in response to enhanced free radicals due to the presence of endotoxin. Despite considerable evidence supporting a role for lipid oxidation in atherogenesis, it has not been possible to identify a robust antioxidant intervention strategy that reproducibly prevents clinical events. The present study was conducted to find out the role of bacterial endotoxin (LPS) in the alteration of tissue anti-oxidant enzymes and to test the hypothesis that hypercholesterolemia alters the inflammatory response in mouse aorta.

MATERIALS AND METHODS

The Institutional Animal Ethical Committee approved all animal protocols. Female Swiss albino mice, (aged 4 weeks) obtained from local registered animal supplier to our department, were randomly assigned to either normal laboratory diet (NLD)-fed groups or a high fat diet (HFD)-fed groups, (n = 6 per group), containing 2% cholesterol and 1% sodium cholate mixed with NLD (19). At 28 weeks of age mice were inoculated intraperitonealy with *E. coli* endotoxin [055:B5, Sigma, USA], at a dose of 1 μg/kg (20) body weight or vehicle containing buffered saline, once per week for 4 weeks. At 32 weeks of age, the animals were sacrificed, after application of the last dose.

Blood was collected by cardiac puncture after anesthetizing the animals with diethyl ether (anesthetic ether), and serum was separated. Serum cholesterol and LDL levels were estimated using bio-clinical kit (Labkit, Chemelex, S.A., Merck, Barcelona, Spain) according to the manufacturers' instruction (21). The liver, heart and aortic tissues were homogenized using a polytron homogenizer. The supernatants obtained after centrifugation of crude homogenates were used to measure catalase, (22) superoxide dismutase (23) and myeloperoxidase (24) enzyme activities. The amount of tissue protein was estimated using dye-binding technique of Bradford (25). Serum TNF-α and IL-6 levels were measured by immuno-enzymatic assay method using the TNF-α [Prepro Tech Inc. Rocky Hill, NJ, USA; Cat # 900-K54] and IL-6 [Pierce Endogen, Rockford, IL, USA; Product #EM21L6] assay kits, specific for mouse, according to the manufacturer's instructions (26, 27).

One-way model I ANOVA was performed between the different groups. Levels of $P<0.05$ was considered significant.

Table 1: Serum Cholesterol, LDL and Cytokine Levels (Mean ±SD)

	Serum Cholesterol (mg/dl)	Serum LDL (mg/dl)	Serum TNF-α (pg/ml)	Serum IL-6 (pg/ml)
NLD + Vehicle	69.18 ± 5.66	24.0 ± 2.33	1112.5 ± 53.03	77.5 ± 2.12
NLD + LPS	66.67±7.32	28.32 ± 3.88	2050.0 ± 70.71**	91.5 ± 3.14 **
HFD + Vehicle	211.3 ±15.78 *	161.08 ± 8.54 *	2125.0 ± 35.55	88.0 ± 8.49
HFD + LPS	137.11 ± 9.12* #	52.59±3.84 * #	2300.0 ± 70.22 **	105.5 ± 2.12 **

* P< 0.001 when compared with respective NLD Groups; # P< 0.001 when compared with HFD-fed vehicle treated groups
** P< 0.01 when compared with respective diet fed vehicle treated groups.

Table 2: Superoxide Dismutase (Units/mg of tissue protein) and Catalase Levels (mmole/min.mg of tissue protein) Mean ± SD

	NLD + Vehicle		NLD + LPS		HFD + Vehicle		HFD + LPS	
	SOD	Catalase	SOD	Catalase	SOD	Catalase	SOD	Catalase
Aorta	2.7 ± 0.2	0.75 ± 0.3	6.7 ± 0.25 #	1.05 ± 0.67	3.8 ± 0.2	1.42 ± 0.55	8.0 ± 0.31 #	1.66 ± 0.85
Liver	7.8 ± 2.5	1.5 ± 0.3	17.5 ± 2.71 #	2.7 ± 0.39 ‡	12.3 ± 2.68 *	1.7 ± 0.4	25.9 ± 4.4 * #	3.1 ± 0.22 ‡
Heart	26.7 ± 5.0	1.3 ± 0.3	38.3 ± 3.56 #	2.0 ± 0.05 ‡	33.3 ± 3.2 *	1.5 ± 0.08	41.7 ± 7.7 * #	2.7 ± 0.08 ‡

* P< 0.05 when compared with respective NLD-fed groups; # P< 0.01 when compared with respective diet-fed vehicle treated groups.
‡ P< 0.05 when compared with respective diet-fed vehicle treated groups.

RESULTS

Serum cholesterol, LDL, IL-6 and TNF-alpha levels of all groups are presented in Table 1. Mice fed with diet supplemented with cholesterol and sodium cholate, i.e. high fat diet (HFD) group had significantly higher (P<0.001) serum cholesterol values as compared to the normal laboratory diet (NLD)-fed groups. It was also noted that HFD-fed animals, but not normal diet-fed animals that were inoculated with lipopolysaccharide (LPS), showed a decrease (P<0.001) in serum cholesterol levels.

Serum LDL levels were significantly increased in mice fed HFD (P<0.001). It was observed that there is a significant decrease (P<0.001) in LDL level after LPS inoculation in mice those were made hyperlipidemic by feeding HFD. In case of NLD-fed animals, slight increase was observed in LPS-treated group as compared to vehicle treated group, but the change is not statistically significant.

After LPS treatment it was seen that serum IL-6 levels were significantly higher than the vehicle treated groups in both NLD and HFD-fed mice (P<0.01). TNF-alpha level was significantly increased in LPS treated NLD-fed mice as compared to vehicle treated NLD-group (P<0.01). But in case of HFD-fed groups, there was no significant difference between LPS and vehicle treated groups.

Table 2 depicts the SOD and catalase levels in tissue homogenates. SOD levels in liver and heart tissue of HFD fed groups were significantly higher than NLD-fed groups (P<0.05). The tissues from LPS-challenged groups also showed significantly higher SOD activity as compared to vehicle treated groups. It was also observed that SOD activity were significantly higher in HFD-fed and LPS-challenged groups than NLD-fed and LPS-challenged groups (P<0.01). In the aortic tissue, there was similarity in results obtained with those of the liver and heart, though the increase in SOD in HFD-fed vehicle treated animals, as compared to NLD-fed vehicle treated animals, were not statistically significant.

Catalase activity in liver and heart tissues showed significant increase in LPS-treated groups irrespective of diet (P<0.05), but in case of aorta the increases were not significant.

Aortic tissue Myeloperoxidase (MPO) content, which is an index of polymorphonuclear neutrophil infiltration, was significantly increased (P<0.001) in LPS-treated groups, irrespective of the type of diet they were fed. The HFD-fed groups also showed significantly higher MPO content when compared to NLD-fed groups (P<0.01) (Table 3).

DISCUSSION

There is a wealth of scientific data coming from *in vitro* studies or from different animal models, supporting the validity of the oxidative hypothesis of atherosclerosis which states that the oxidative modifications of lipoproteins is a pivotal event in the evolution of atherosclerotic plaques. A corollary of this hypothesis is that antioxidant enzymes should therefore prevent LDL oxidation and protect against the development of atherosclerosis (28).

Our study shows that total cholesterol levels were significantly elevated in mice fed a high fat diet. Accordingly LDL level in those mice were also greater. But we observe that

Table 3: Myeloperoxidase Levels (Units/min.mg of tissue protein) Mean ± SD

	NLD + Vehicle	NLD + LPS	HFD + Vehicle	HFD + LPS
Aorta	10.8 ± 2.87	32.6 ± 4.12 #	20.5 ± 4.79 *	53.2 ± 9.41 * #

* P< 0.001 when compared with respective NLD Groups; # P< 0.001 when compared with respective diet fed vehicle treated groups

endotoxemia causes a decline in serum LDL in hyperlipidemic mice. Hypercholesterolemia has been associated with enhanced oxidative stress related to increased lipid peroxidation. Therefore the inhibition of oxidative stress under hypercholesterolemic conditions is considered to be an important therapeutic approach and many efforts have been made to identify the antioxidative pathways. Endotoxemia is also accompanied by significant changes in the reductive-oxidative balance of critical target organs (29). Liver is the main site of lipoprotein metabolism and migration of modified lipoproteins into vascular endothelium leads to inflammatory responses. Thus we hypothesized that the antioxidant enzyme in critical target organs, mainly the liver, heart and aorta, may be upregulated by the administration of *E. coli* LPS in response to enhanced free radicals due to the presence of endotoxin, by LPS itself or by cytokines activated by LPS. A previous study had compared the effects of redox imbalance on hepatic responses of mice to *E. coli* versus purified endotoxic LPS (30). Here we report enhanced SOD activity in mouse liver, aorta and also heart tissue homogenates after LPS administration in the NLD-fed mice and it was significantly increased in the HFD-fed groups. A large number of studies demonstrate the protective effect of SOD in various models of endotoxic shock; furthermore there is a large amount of evidence to show that production o reactive oxygen species such as O_2^-, H_2O_2, and HO^-, occurs at the site of inflammation and contribute to tissue damage. Therefore, enhanced tissue SOD during hypercholesterolemia and endotoxemia may be a preventive measure of the host to handle the superoxide anion load after bacterial LPS administration.

We found elevated liver catalase after LPS administration in HFD-fed mice. It may be suggested that after LPS administration in the HFD-fed mice there may be elevated H_2O_2 in the liver, to scavenge those increased oxidant burden liver tissue have more catalase expression. A previous study (31) also supported the notion that LPS provoked an increase catalase activity in mice and this might be related with the generation of ROS such as H_2O_2 associated with endotoxemia. Clinically antioxidant enzymes are believed to counteract ROS and reduce the incidence of coronary artery disease. Selective antioxidants are, thus, possible candidates for anti-atherosclerotic clinical trials.

There is evidence that MPO promotes LDL oxidation *in vivo* (32). The reduction in the level of LDL after endotoxemia can possibly be due to elevated MPO levels, found by us, that lead to modification of LDL. Since LPS injected mice given a high fat diet displayed significantly elevated inflammatory response, it clearly indicates that both endotoxemia and high fat diet *per se* were significant stimuli for atherosclerosis induction in this mouse model. The mechanism underlying the enhanced tissue antioxidant enzymes and concomitant MPO activity in the aorta needs to be clarified.

Since LPS exposure induces the production of inflammatory mediators that have been implicated in atherogenesis, endotoxin may therefore be linked to atherosclerosis, although this hypothesis is at present very controversial (33). In our study, after endotoxin administration, cytokines are released systemically (TNF, IL-6) and in that setting may become crucial mediators accelerating the progression of inflammatory disease during hyperlipidemia. This elevated systemic cytokines (TNF, IL-6) may enhance PMN adhesion to endothelial surfaces, degranulation production of superoxide anion, release of lysozyme and H_2O_2 and chemotaxis suggesting implication in increased inflammatory response.

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