Introduction

Overproduction of nitric oxide (NO) has been contributed to tissue injury in inflammatory conditions. One mechanism by which NO can contribute to tissue injury is by its diffusion–controlled reaction with superoxide (O_2^-) to produce peroxynitrite ($ONOO^-$), a potent oxidant thought to be a key mediator of NO mediated tissue injury in a dose–dependent manner in atherosclerosis, congestive heart failure and other disease states involving inflammatory oxidative stress [1-3]. NO is formed from the semi-essential amino acid L-arginine by the action of three isoforms of the nitric oxide synthase (nNOS, cNOS, iNOS). iNOS produces high amount NO after exposure to proinflammatory cytokines [3-5]. $ONOO^-$ reacts with the phenolic ring of tyrosine to form a stable product, 3 nitrotyrosine (3-NT). 3-NT has been widely used as a marker for the detection of $ONOO^-$ formation in vivo [6-8]. Asymmetric dimethylarginine (ADMA) is an L-arginine analog that serves an endogenous inhibitor of both cNOS and iNOS. ADMA is involved in the pathophysiology of vascular disease and has been demonstrated to be not only a marker of endothelial dysfunction, but also a novel cardiovascular risk factor. ADMA has been reported competitively inhibit NO elaboration by displacing L-arginine from NOS [1, 9, 10]. The levels of ADMA and NO are dependent on the prevailing L-arginine concentrations. Clinical and experimental evidences have suggested that elevation of ADMA can cause a relative L-arginine deficiency, even in the presence of normal L-arginine level. In addition to producing NO, NOS can catalyze O2- formation in the presence of low L-arginine level [1,11]. The role of ADMA and L-arginine levels in the regulation of ONOO- production in the heart after endotoxin administration has not yet been well explored. The aim of our study was to examine the effect of endotoxin administration on the heart levels of L-arginine, ADMA and 3-NT and to investigate the relationship between ADMA, L-arginine level and peroxynitrite generation in guinea pigs.

Material and Methods

Animals and study protocol

Following ethics committee approval, all experiments was performed with 20 male Dunkin Hartley guinea pigs weighing 500- 600 g. The animals were randomly divided into two groups. Group I (n=10) served as a control group and was given only saline solution. Group II (n=10) served as an endotoxemia group and was administrated intraperitoneally lipopolysaccharide (LPS) (Escherichia coli LPS serotype O111:B4, from sigma 4mg/kg.).

Measurement of ADMA and L-arginine

Measurement of ADMA and L-arginine level were accomplished by high pressure liquid chromatography (HPLC) using the method described by Momohora, Chen and Azuma et al. [12,13,14]. 300 mg tissue was homogenized in 3 ml phosphate buffer (pH: 6.5). The homogenate was centrifuged at 10000 g for 20 min at 4 0C. Then 60 mg tricarboxylic acid was added to the 1 ml supernatant and the mixture was left in an ice-bath for 10 min. The precipitated protein removed by centrifugation at 3000 g. for 15 min. The supernatant which was filtered through a 0.2 µm filter was mixed with 100 µL of derivatization reagent (prepared by dissolving 10 mg o-phtaldialdehyde in 0.5 ml of methanol, 2 ml 0.4 M borate buffer (pH:10.0) and 30 µL of 2-mercaptoethanol) and then injected into the chromatographic system. Separation of ADMA and L-arginine levels were achieved with 150 x 4 mm I.D. Nova-pak C18 column with a particle size of 5 µm (waters, Milipore, Milford MA, USA) using 50 mM sodium acetate (pH:6.8), methanol and tetrahydrofurane as mobile phase (A, 82:17:1; B, 22:77:1) at flow rate 1.0 ml/min. The areas of peaks detected fluorescent detector (Ex: 338 nm; Em: 425) were used for quantification ADMA and L-arginine levels in heart. The analytical variability of the method was less then 7%, and the detection limit of the assay was 0.1 µM.

Measurement of Tyrosine Nitration

Tissue homogenenates were prepared according to the method described by Kamisaki et al [15]. Briefly, 0.5 g heart tissue was homogenized in 1.5 ml of 50 mM potassium phosphate buffer (pH: 7.2) and then centrifuged for 5 min at 600 g. Following the acid hydrolysation of the precipitate, it was evaporated under nitrogen. After 1 ml distilled water adding, 10 µL of sample was applied to HPLC. All samples were analysed by HPLC with ECD using the method described by Maruyama et al [16]. For 3-NT detection a Macrotech C-18 analytical column (50 x 1 mm) was used with 50 mM phosphoric acid / 50 mM citric acid / %5 (v/v) methanol (pH:3.1) as the mobile phase. Applied potentials were -850 mV for the reduction and 600 mV for the oxidation detection cell.

Statistical analysis

Results were expressed as mean ± standard deviation (SD). 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 analyse the significance of the difference between control and experimental groups. For tests of significance a p value of less than 0.05 was considered to be significant.

Results

Endotoxin administration did not change levels of ADMA in heart tissues compared to the control group. After endotoxemia levels of L-arginine in heart tissues were reduced but were not statistically significant. 3-NT levels in the heart tissues significantly increased ($p < 0.05$) after endotoxin given. The levels of ADMA, L–Arginine and 3-NT in heart tissues are shown in Table 1.

Table 1. The levels of ADMA, L–Arginine and 3-NT in heart tissues.

	ADMA (µmol /g tissue)	L-Arginine (nmol /g tissue)	3-NT (nmol / g tissue)
Control group	0.46 ± 0.0061	37.2 ± 6.8	0.94 ± 0.21
Endotoxin group	0.48 ± 0.094	33.5 ± 4.3	1.24 ± 0.17*

Results were expressed as mean ± standard deviation (SD)
* p <0.05

Discussion

Recent studies suggested that exposure animal hearts or cardiac myocytes to bacterial endotoxin (LPS) or proinflammatory cytokines enhanced NO level [2, 4, 6, 17]. In cells NO arises from the enzymatic oxidation (by NO synthase) of guanidino nitrogen of one terminal of L-arginine. ONOO- is reaction product formed by the interaction between NO and O2- and it has also been implicated as a toxic mediator in the pathophysiology of endotoxemia in atherosclerosis [1, 2]. ONOO- reacts with tyrosine to form, a stable product, 3-NT [3, 6]. ADMA, a naturally occurring analog of L-arginine, competitively inhibits NO production by NOS. L-arginine is an important substrate for both NO and ADMA production. A link between ADMA and inflammation has been demonstrated in the

prediction of cardiovascular events [18]. We examined the relationship between ONOO- production and levels of ADMA, L-arginine in heart tissues after endotoxin administration. The present study for the first time demonstrated increased 3-NT level in endotoxemic heart tissues, but concurrent with unchanged ADMA production. In addition to this study provides evidence for decreased L-arginine level but no significant. In a previously our study we found elevated plasma ADMA levels in same animals with endotoxemia, as well as decreased L-arginine levels and declined the ratio of Larginine/ ADMA, but NO levels did not change [19]. In contrast to the plasma, in this study, levels of ADMA and L-arginine in heart tissues did not change. But after endotoxemia in the heart tissues of same animals, ONOO- production was high. It may be dependent on the mechanism found in the heart exposed to LPS. Our results may be consistent with previous observation. Investigators have demonstrated that the 3-NT levels were increased in LPS-treated hearts (4 mg/kg). The other investigators examined the cytotoxic effect of iNOS-generated NO in cultured cardiac myocytes treated with LPS. They showed that the cytokines caused nitrotyrosine formation in the injured myocytes [2-4]. In our study, while the levels of 3-NT in animal heart tissues increased with endotoxemia, ADMA and L-arginine levels decreased but this decrease was not significant. There was no correlation between ADMA, L-arginine and 3-NT. These results were in agreement with previous observations in vitro that ADMA preferentially inhibited cNOS isoform, while having minimal effect on NO production by iNOS [1]. In the other in vivo study, it has been reported that E. coli mediated endotoxemia decreases the plasma arginine level but ADMA did not change [19]. Ueda et al. demonstrated exogenous administration of NO donors dose-dependently increased NO synthesis in the cultured media but had no effect on ADMA and L-arginine levels [20]. In conclusion we could not find a link between ADMA, L-arginine concentrations and ONOO- production in the heart after endotoxin administration. More researches may be needed to elucidate these results using different dose and time after LPS administration.

References

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