

Investigation of nitric oxide-ADMA pathway of liver and kidney in endotoxemia

[Endotoksemide karaciğer ve böbrek dokularında nitrik oksit-ADMA yolağının araştırılması]

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ABSTRACT

Purpose: We aimed to investigate the relationship between L-arginine-nitric oxide (NO) and L-arginine-asymmetric dimethylarginine (ADMA) pathways in kidney and liver during endotoxemia.

Material and Methods: In the present study, twenty adult male guinea pigs were randomly divided into two groups; control and endotoxemia. Endotoxemia was induced by a single intraperitoneally injection of lipopolysaccharide (LPS, 4 mg/kg). After six hours of incubation, when highest blood level of endotoxin was attained, the animals were sacrificed, and kidney and liver tissue samples were collected. The amounts of ADMA and L-arginine were measured by HPLC, and arginase activity and levels of reactive nitrogen oxide species (NOx) which are stable end products of NO were measured spectrophotometrically.

Results: LPS administration significantly decreased ADMA and L-arginine levels, and increased arginase activity and NOx levels compared to control group in liver. As for kidney, LPS administration significantly elevated ADMA levels and arginase activity, and decreased NOx levels compared with control group. But, L-arginine levels did not change in kidney during endotoxemia.

Conclusion: It has been concluded that L-arginine-NO, L-arginine-ADMA pathways and arginase activity were important factors in endotoxin mediated liver and kidney tissue impairment. The changes observed in L-arginine-NO and L-arginine-ADMA pathways were opposite of each other during endotoxemia. We believed that this situation was the result of functional differences in these tissues.

Key Words: Endotoxin, ADMA, Arginine, NOx, Liver, Kidney.

ÖZET

Amaç: Çalışmamızda, endotoksemi esnasında karaciğer ve böbrek dokularındaki L-arginin-nitrik oksit (NO) ve L-arginin-asimetrik dimetilarjinin (ADMA) yolağları arasındaki ilişkinin araştırılması amaçlanmıştır.

Gereç ve Yöntemler: Deneyde 20 adet erkek Dunkin Hartley kobay, kontrol ve endotoksemi grupları olmak üzere rastgele 2 gruba ayrılmıştır (n=10). Endotoksemi tek doz, 4 mg/kg lipopolisakkarit (LPS) in intraperitoneal enjeksiyonu ile oluşturulmuştur. Uygulamadan 6 saat sonra, endotoksin konsantrasyonu kanda en yüksek seviyeye ulaştığında, kobaylar anestezi altında feda edilmiş, böbrek ve karaciğer dokuları uygun koşullarda alınmıştır. Dokulardaki L-arginin ve ADMA düzeyleri HPLC ile, arjinaz aktivitesi ve NO' nun stabil son ürünleri olan reaktif azot oksit ürünleri (NOx) konsantrasyonu spektrofotometrik olarak ölçülmüştür.

Bulgular: LPS uygulaması, karaciğer dokusundaki ADMA ve L-arginin seviyelerini kontrol grubuyla karşılaştırıldığında istatistiksel olarak anlamlı şekilde azaltırken, NOx düzeyini ve arjinaz aktivitesini belirgin şekilde arttırmıştır. Böbrek dokusunda ise, LPS uygulamasından sonra ADMA seviyesi ve arjinaz aktivitesi anlamlı şekilde artmış, NOx düzeyi azalmıştır. Buna karşılık, endotoksemi esnasında böbrek dokusu L-arginin düzeyinde istatistiksel olarak anlamlı bir değişiklik saptanmamıştır.

Sonuçlar: Karaciğer ve böbrek dokularında, L-arginin-NO, L-arginin-ADMA yolağları ve arjinaz aktivitesi endoksinlerin yol açtığı hasarda önemli bir rol oynamaktadır. Endotoksemide, böbrek ve karaciğer dokularındaki L-arginin-NO ve L-arginin-ADMA yolağlarında meydana gelen değişiklikler birbirinin tamamen karşıtıdır. Bu durumun, söz konusu organların fonksiyonel farklılıklarından kaynaklandığı düşünülmektedir.

Anahtar Kelimeler: Endotoksin, ADMA, Arjinin, NOx, Böbrek, Karaciğer.

Introduction

The L-arginine-nitric oxide (NO) pathway has been recognized to play critical roles during infections and inflammation. Recently, it is also known that endogenous L-arginine analogues might play a regulatory role in the L-arginine-NO pathway [1,2]. Asymmetric dimethylarginine (ADMA) is an endogenous inhibitor of all isoforms of NO synthases (NOS). It is formed from L-arginine residues in proteins, and it competes with L-arginine for each of three isoforms of NOS [3,4]. The concentration of ADMA is regulated by urinary excretion. Besides elimination of ADMA by kidney, ADMA is degraded by dimethylarginine dimethylaminohydrolase (DDAH), which is highly expressed in liver, but also present in kidney, pancreas and endothelial cells [1,5]. On the other hand, the production of L-arginine is executed by the successive action of small intestine and kidney (the gut-kidney axis). L-citrulline is produced from L-glutamine in small intestine, and L-citrulline is released the circulation and taken up by kidney where it is converted to L-arginine [6-8]. In addition, arginase is an enzyme that catabolizes L-arginine to L-ornithine and urea, and liver has a high arginase activity. There are at least two distinct isoforms of arginase present. Arginase I is a cytosolic enzyme that is highly expressed in liver, and constitutes a majority of total arginase activity. In contrast, arginase II is a mitochondrial enzyme that is expressed at lower levels in kidney, brain, intestine and lung [9].

The understanding of relationship between L-arginine-NO and L-arginine-ADMA pathways in liver and kidney during endotoxemia is important. In our previous study, we reported that there is a possible interaction between plasma L-arginine, ADMA levels and increased oxidative stress in LPS-treated guinea pigs [10]. However, a little is known about these pathways, and their comparing in liver and kidney during endotoxemia. Thus, in the present study, we aimed to investigate the relationship between L-arginine-NO and L-arginine-ADMA pathways in kidney and liver in guinea pigs after administration of endotoxin, to measure the amount of L-arginine and arginase activity which may be responsible for the central regulation of these pathways, and to compare the results of kidney and liver.

Material and methods

Chemicals

LPS (0111:B4 from *Escherichia coli*) was purchased from Sigma-Aldrich (St. Louis, MO, USA). All other chemicals used were of the highest analytical grade, and purchased from Merck (Darmstadt, Germany) or Sigma-Aldrich (St. Louis, MO, USA).

Animals and Study Protocol

This study was carried out in accordance with the regulations of Animal Experimentation Ethics Committee at

Gulhane Military Medical Academy. Twenty adult male Dunkin Hartley guinea pigs weighing 500-600 g in were obtained from Laboratory Animal Breeding and Experimental Research Center at Gulhane Military Medical Academy. They were provided with standard rat chow and tap water *ad libitum* until the experiments. Guinea pigs were randomly divided into two groups at 10 animals each; control and endotoxemia. Endotoxemia was induced by a single intraperitoneally (i.p.) injection of LPS (4 mg/kg body weight) [11]. LPS was prepared daily, dissolved in nonpyrogenic sterile saline, and warmed to body temperature (approximately 37°C) before the injection. Control group was i.p. injected with same volume of 0.9%NaCl. After six hours of incubation, when highest blood level of endotoxin was attained, all animals were anesthetized with intramuscular ketamine (60 mg/kg) and xylazine (10 mg/kg), and sacrificed. The livers and kidneys were removed, and immediately frozen in liquid nitrogen, and then stored at -80°C until assay.

L-arginine and ADMA Analysis

Measurement of L-arginine and ADMA levels were accomplished by high performance liquid chromatography (HPLC) using the method described by various workers [12-14]. In brief, 300 mg tissue in 3 ml phosphate buffer (pH 6.5) was homogenized at maximum speed for 20 s. The homogenate was centrifuged at 10 000 g for 20 min at 4°C. Then 60 mg thio carboxylic acid was added to the 1 ml supernatant, and the mixture was left in an ice-bath for 10 min. The precipitated protein was removed by centrifugation at 3 000 g for 15 min. The supernatant was filtered through a 0.2 µm filter, and mixed with derivatization reagent (prepared by dissolving 10 mg o-phthaldialdehyde in methanol, 0.4 M borate buffer, pH 10.0 and 2-mercaptoethanol), and then auto-injected into the chromatographic system. Separation of L-arginine and ADMA were achieved with I.D. Nova-pak C18 column (particle size 5 µm, 150 × 4 mm). Mobile phase was 50 mM sodium acetate (pH 6.8), methanol and tetrahydrofuran (A, 82:17:1; B, 22:77:1, v/v/v). The flow rate was 1 ml/min. Fluorescence detector was set at 338 nm and 425 nm for excitation and emission. The variability of the method was less than 7%, and the detection limit of the assay was 0.1 µM. Tissue L-arginine and ADMA concentrations were expressed as nmol/g tissue and µmol/g tissue, respectively.

NOx Measurement

Levels of reactive nitrogen oxide species (NOx) which are stable end products of NO were given as sum of nitrite and nitrate. The amount of nitrate was determined by a single step enzymatic method constituting of the reduction of nitrate by the nitrate reductase enzyme in the presence of NADPH and the measurement of the decrease in absorbance at 340 nm [15]. Nitrite levels were measured by the Griess reaction. Sodium nitrite and nitrate solutions (1, 10, 50, 100 µmol/L) were used

as standards, and tissue NOx content was expressed as $\mu\text{mol/g}$ tissue [16].

Determination of Arginase Activity

The thiosemicarbazide-diacetylmonoxime urea (TDMU) method was used to measure arginase activity with spectrophotometer. This method based on the determination of urea produced as a result of reaction of L-arginine-arginase, and tissue arginase activity was expressed as U/mg protein/h [17].

Protein Measurement

Protein levels of liver and kidney tissues were assessed by Lowry method [18].

Statistical Analysis

Statistical analysis was performed using a software program (SPSS 16.0 for windows, Chicago, IL, USA). All values were expressed as mean \pm standard deviation. The nonparametric Mann-Whitney U test was used to analyze the significance of the differences between control and endotoxemia groups. For tests of significance a *p* value of 0.05 or less was considered significant.

Results

As shown in Table 1, NOx level of liver in endotoxemia group was significantly higher than that of control group (control 1.92 ± 0.24 $\mu\text{mol/g}$ tissue, endotoxemia 2.59 ± 0.37 $\mu\text{mol/g}$ tissue). LPS administration significantly decreased both ADMA and L-arginine levels compared with

control group ($p < 0.05$). Liver arginase activity was significantly elevated after LPS treatment in endotoxemia group compared with control level (368.4 ± 81.2 U/mg protein/h and 294.2 ± 15.0 U/mg protein/h, respectively).

In contrast to liver, as seen in Table 2, LPS administration significantly decreased renal NOx level compared to control group ($p < 0.05$). L-arginine concentration was 41.27 ± 5.40 nmol/g tissue in control group and 49.07 ± 8.71 nmol/g tissue after LPS administration, and the difference between them was not statistically significant. ADMA level and arginase activity in endotoxemia group was significantly higher than that of control group ($p < 0.05$).

Discussion

The L-arginine-NO pathway has been recognized to play critical roles during pathological conditions such as infections and inflammation [1,2]. Recently, it was also reported that endogenous L-arginine analogues may play a regulatory role in the L-arginine-NO pathway. ADMA is an endogenous inhibitor of all isoforms of NOS by competing with L-arginine for binding to the enzyme's catalytic domain [3,4]. A link between ADMA and inflammation has been shown previously [10]. In the present study, endotoxemia was induced by 4 mg/kg i.p. injection of LPS in guinea pigs. Because inducible isoform of NOS (iNOS) was maximally induced by endotoxin at 6th h after treatment [19,20], liver and kidney tissues were removed, and levels of ADMA, L-arginine, NOx and arginase activity were measured after LPS

Table 1. NOx, ADMA, L-arginine levels and arginase activity in liver

	NOx ($\mu\text{mol/g}$ tissue)	ADMA ($\mu\text{mol/g}$ tissue)	L-arginine (nmol/g tissue)	Arginase (U/mg protein/h)
Control	1.92 ± 0.24	0.78 ± 0.21	19.97 ± 4.65	294.2 ± 15.0
Endotoxemia	$2.59 \pm 0.37^*$	$0.35 \pm 0.09^*$	$11.22 \pm 1.01^*$	$368.4 \pm 81.2^*$

Data was shown as mean \pm standard deviation.

* $p < 0.05$ compared to control group.

Table 2. NOx, ADMA, L-arginine levels and arginase activity in kidney

	NOx ($\mu\text{mol/g}$ tissue)	ADMA ($\mu\text{mol/g}$ tissue)	L-arginine (nmol/g tissue)	Arginase (U/mg protein/h)
Control	2.10 ± 0.50	0.624 ± 0.08	41.27 ± 5.40	8.41 ± 1.89
Endotoxemia	$1.54 \pm 0.41^*$	$1.059 \pm 0.14^*$	49.07 ± 8.71	$15.82 \pm 3.70^*$

Data was shown as mean \pm standard deviation

* $p < 0.05$ compared to control group.

administration at 6th. In this study, we found that LPS administration significantly elevated NO_x level and decreased ADMA and L-arginine levels in liver. In addition, arginase activity was significantly high compared to control group. Tabuchi et al. [20] reported that L-arginine synthetic enzyme activities are high, and a large amount of L-arginine is synthesized in hepatocytes. However, the synthesized L-arginine is completely degraded by the high activity of arginase I. In endotoxic shock, iNOS is highly induced in hepatocytes, and L-arginine is utilized for a high output production of NO. It was determined that there is a potential link between inflammation and metabolism of ADMA [20,21]. Liver plays an important role in eliminating ADMA from the circulation, and endotoxemia stimulates this capacity [22]. The probable explanation for the reduced ADMA level is an intense catabolism by DDAH, which is present in liver in high amount [5,23,24]. Siroen et al. [24] reported that DDAH activity increased after LPS administration in animal models. Our results were compatible with these evidences. Our findings suggested that these conditions were characterized by overproduction of NO via increased iNOS activity during endoxemia. We also presented data on arginine handling of liver as L-arginine was the key amino acid in the L-arginine-NO and L-arginine-ADMA pathways.

When compared with liver, kidney showed a quite different handling of ADMA and L-arginine. In contrast to the liver, decreased NO_x concentration, increased ADMA and unchanged L-arginine levels were found after administration of LPS in kidney. Arginase activity in kidney also increased as liver after administration of endotoxin. Hallemeesch et al. [7] reported that although increased systemic NO production during endotoxemia, they were not able to detect renal NO production in LPS-treated mice. Two possible causes of NO deficiency were substrate L-arginine limitation, and increased levels of endogenous inhibitors of NO synthases particularly ADMA [2]. In agreement with these results, we found that ADMA level increased, but L-arginine level didn't change in renal tissues. In the present study, the cause of the unchanged arginine level might be due to its usage in synthesis of ADMA or degradation by arginase. Luiking et al. [25] reported that denovo renal L-arginine production didn't change during early endotoxemia in mice. Cichy et al. [26] suggested that endotoxemia is commonly associated with acute renal failure, and this renal failure may be result of vasoconstriction in the kidney. In the other hand, Wang et al. [27] and Zhang et al. [28] reported that acute renal failure during endotoxemia is associated with increased NO. Decreased NO level was important for preservation of renal function in endotoxemia [29]. There are strong evidences that ADMA and L-arginine might be uptake by y⁺ transporter in kidney, and expression of this transporter was significantly increased by LPS injection. In addition, LPS infusion resulted in a reduced renal elimination of ADMA [26,30].

It was reported that elevated tissue levels of ADMA in kidney disease were functions of both reduced renal excretion and catabolism by DDAH [31]. Nijveldt et al. [30] showed that DDAH activity decreased in endotoxemia. On the other hand, the present results showed that arginase was induced by endotoxin administration in kidney as well as liver. In the previous studies, it was suggested that arginase activity could regulate the rate of induced NO generation through L-arginine availability for NO synthesis [32,33]. In kidney, on the other hand, the iNOS mRNA and protein were induced by LPS. But arginase and iNOS were induced in the same cells, arginase II might compete with iNOS for L-arginine, and down regulate NO production [33, 34]. In this experiment indicates the possibility arginase activity increased, but iNOS activity decreased after endotoxin administration.

In conclusion, the results of present study indicate L-arginine-NO, L-arginine-ADMA pathways and arginase activity at 6th h after 4 mg/kg endotoxin injection might be important factor in the endotoxin-mediated liver and kidney damage. The changes observed in L-arginine-NO and L-arginine-ADMA pathways were opposite of each other except arginase activity in liver and kidney. During endotoxemia, ADMA decreased and NO_x increased in liver whereas ADMA increased and NO_x decreased in kidney. We believed that this situation was the result of functional differences in these tissues.

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Conflict of Interest

Authors have no conflict of interest.

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