Acute Escherichia coli endotoxaemia decreases the plasma L-arginine/asymmetrical dimethylarginine ratio in humans

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ABSTRACT

Acute inflammation impairs vascular function. Based on the association between endothelial dysfunction and plasma concentrations of L-arginine and the endogenous nitric oxide synthase inhibitor ADMA (asymmetrical dimethylarginine), we hypothesized that the ratio between L-arginine and ADMA could be affected by experimental inflammation. Plasma concentrations of L-arginine, ADMA and SDMA (symmetrical dimethylarginine) were studied at baseline and 3.5 h after intravenous administration of Escherichia coli endotoxin [LPS (lipopolysaccharide), 20 units/kg of body mass; n = 8] or placebo (n = 9) in healthy males. L-Arginine and dimethylarginines were quantified after solid-phase extraction by reversed-phase HPLC. Body temperature, heart rate and leucocyte count increased after LPS administration (P < 0.01 for all). LPS administration decreased plasma concentrations of L-arginine from 66 µmol/l [95 % CI (confidence interval): 56, 88] at baseline to 48 µmol/l (CI: 40, 60) after 3.5 h (P < 0.02), but did not affect ADMA and SDMA concentrations. Consequently, the L-arginine/ADMA ratio declined significantly from a median of 159 (CI: 137, 193) to 135 (CI: 103, 146); a decrease of 25 (CI: −68, −13; P < 0.02). L-Arginine, ADMA, SDMA and the L-arginine/ADMA ratio remained constant over time in controls. Acute inflammation reduces the L-arginine/ADMA ratio which could contribute to impaired vascular function.

INTRODUCTION

Elevated circulating concentrations of ADMA (asymmetrical dimethylarginine), an endogenous inhibitor of NOS (nitric oxide synthase) [1], or a decreased L-arginine/ADMA ratio have been described in patients with endothelial dysfunction, such as renal failure [1,2], hypercholesterolaemia [3], hypertriglyceridaemia [4], Type II diabetes [5], women with previous gestational diabetes [6], heart failure [7], pre-eclampsia [8,9] and peripheral arterial occlusive disease [10].

Systemic inflammation, as reflected by increased plasma levels of tumour necrosis factor-α and CRP (C-reactive protein), is detectable in acute coronary syndromes and associated with the clinical outcome [11–15]. A link between ADMA and inflammation has been demonstrated recently by a relationship between ADMA and CRP in the prediction of cardiovascular events in patients on haemodialysis [16].

Several studies have shown that vasodilation and endothelial dysfunction are present after systemic administration of low doses of Escherichia coli endotoxin [LPS (lipopolysaccharide)] in healthy men [17,18]. This model reproduces many of the pathological aspects of severe inflammation, such as haemodynamic changes [18,19] and coagulation activation [20]. Based on the

Key words: L-arginine, dimethylarginine, endotoxin, Escherichia coli, HPLC, inflammation.

Abbreviations: ADMA, asymmetrical dimethylarginine; CRP, C-reactive protein; LPS, lipopolysaccharide; SDMA, symmetrical dimethylarginine; (i)NOS, (inducible) nitric oxide synthase; CI, confidence interval.

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association between L-arginine, ADMA and endothelial dysfunction, we examined the effect of LPS administration on the plasma concentrations of L-arginine and dimethylarginines in healthy young men.

METHODS

The study was approved by the Ethics Committee of the University of Vienna and complied with the Declaration of Helsinki, including current revisions and the Good Clinical Practice guidelines.

Seventeen healthy young men, with normal laboratory and renal function tests, and no cardiovascular medical history were included. The subjects were studied in fasting conditions according to a double-blind, randomized parallel-group design. Eight subjects (27 ± 1 years) received E. coli endotoxin (LPS; intravenous bolus, 20 units/kg of body mass), and nine subjects (27 ± 2 years), received placebo and served as time controls. Blood was drawn before and 3.5 h after LPS or placebo administration and put into tubes containing EDTA, and plasma supernatants were stored at −80 °C until batch analyses. L-Arginine and dimethylarginine analyses in plasma were done while blinded to the treatment allocation of the subjects.

L-Arginine, ADMA and SDMA (symmetrical dimethylarginine)

For measurement of L-arginine, ADMA and SDMA, plasma was subjected to cation exchange solid-phase extraction using an Oasis MCX column (30 mg, 1 ml; Waters, Milford, MA, U.S.A.) according to a slightly modified method described previously [21]. After application of the samples and the internal standard monomethylarginine (40 μM) diluted in PBS (pH 7.2), 1 ml of 100 mM HCl and 1 ml of methanol were administered for washing. Subsequently, the basic amino acids were eluted with 1 ml of concentrated ammonia/water/methanol (1:4:5, by vol.). Thereafter, the eluent was dried by evaporation under vacuum (1 mbar, where 1 bar = 105 Pa) at 60 °C. The dried extract was redisolved in distilled water and mixed 1:1 with derivatization reagent (10 mg of o-phthalaldehyde in 200 μl of methanol and 10 μl of 3-mercaptopropionic acid diluted 1:50 with 200 mM borate buffer, pH 9.5). The sample was separated on a Symmetry C18 column (3.9 mm × 150 mm, 5 μm, 100 Å pore size) protected by a Sentry C18 precolumn from Waters using HPLC. The system consisted of 2 PU-980 HPLC gradient pumps, an AS-950 autosampler and a fluorescence detector model 821-FP (Japan Spectroscopic Co., Osaka, Japan). L-Arginine, ADMA, SDMA and the internal standard were eluted with 50 mM sodium phosphate buffer (pH 6.5; solvent A) containing 8.7 % acetonitrile at a flow rate of 1.1 ml/min. After elution of the last analyte strongly retained compounds were eluted with 50 % acetonitrile/water (1:1, v/v) for 3 min. The column was reconditioned with 100 % solvent A for 6 min before injection of the next sample. Detection was performed with excitation set at 340 nm and emission at 445 nm. A calibration curve was constructed with standards treated in the same way as plasma samples. The coefficients of variation for inter- and intra-sample variations tested with a pooled plasma sample were below 3 % for all analytes. The detection limit for dimethylarginines was 0.04 μmol/l.

Laboratory and physiological parameters

Standard laboratory methods were used for measurement of leucocyte count in the ISO certified laboratory at the Clinical Institute for Medical and Chemical Laboratory Diagnostics, AKH Wien, Austria. Tympanic temperature (Thermoscan pro, Braun GmbH, Kronberg, Germany), blood pressure and heart rate (Hewlett Packard CMS patient monitor, Palo Alto, CA, U.S.A.) were monitored non-invasively at frequent intervals.

Drugs and chemicals used

E. coli endotoxin was obtained from U.S. Pharmacopeial Convention (Rockville, MD, U.S.A.). N0,N0-dimethyl-L-arginine (ADMA) and N0,N0-dimethyl-L-arginine (SDMA) were obtained from Calbiochem (Darmstadt, Germany). All other materials were from Sigma–Aldrich (St. Louis, MO, U.S.A.).

Statistics

Clinical parameters were normally distributed, and paired and unpaired Student’s t test used for assessment of within and between group differences accordingly. Non-parametric statistics were applied for L-arginine, ADMA and SDMA. Differences within and between groups were analysed by Wilcoxon-matched pairs test and the Mann–Whitney U test respectively, using the Statistica® software package (Release 6, StatSoft, Tulsa, OK, U.S.A.). A P value of < 0.05 was defined as the level of significance.

RESULTS

No side effects apart from the expected flu-like symptoms were reported in response to LPS administration. These transient symptoms were only mild, and all subjects were discharged symptom-free from our unit after approx. 9 h. No differences between groups were detectable at baseline. Clinical and laboratory data are summarized in Table 1.

Body temperature and leucocyte count increased 3.5 h after LPS administration (P < 0.01 versus baseline). LPS significantly increased heart rate (P < 0.01 versus
Table 1  Outcome parameters in subjects receiving E. coli endotoxin (LPS, 20 units/kg of body mass; n = 8) and time controls (n = 9) at baseline and after 3.5 h
Results are expressed as the means ± S.E.M. *P < 0.01 versus baseline, †P < 0.05 versus controls; Student's t test.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Baseline</th>
<th>3.5 h</th>
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<tbody>
<tr>
<td>Temperature (°C)</td>
<td>LPS 35.9 ± 0.1</td>
<td>37.2 ± 0.3*†</td>
</tr>
<tr>
<td></td>
<td>Controls 36.2 ± 0.1</td>
<td>36.6 ± 0.1</td>
</tr>
<tr>
<td>Heart rate (beats/min)</td>
<td>LPS 68 ± 3</td>
<td>88 ± 6*†</td>
</tr>
<tr>
<td></td>
<td>Controls 65 ± 4</td>
<td>62 ± 4</td>
</tr>
<tr>
<td>Systolic blood pressure (mmHg)</td>
<td>LPS 122 ± 3</td>
<td>117 ± 4</td>
</tr>
<tr>
<td></td>
<td>Controls 122 ± 2</td>
<td>121 ± 5</td>
</tr>
<tr>
<td>Diastolic blood pressure (mmHg)</td>
<td>LPS 66 ± 2</td>
<td>61 ± 3</td>
</tr>
<tr>
<td></td>
<td>Controls 66 ± 2</td>
<td>64 ± 4</td>
</tr>
<tr>
<td>Leucocyte count (10⁹/litre)</td>
<td>LPS 5.24 ± 0.26</td>
<td>9.36 ± 0.58*†</td>
</tr>
<tr>
<td></td>
<td>Controls 5.31 ± 0.27</td>
<td>5.28 ± 0.32</td>
</tr>
</tbody>
</table>

baseline) and slightly decreased systolic and diastolic blood pressure, which did not reach the level of significance. All parameters were unchanged over time in controls.

The plasma concentration of L-arginine was significantly lower after LPS administration {median decrease 15 μmol/l [95% CI (confidence interval): 6, 38]; P < 0.02}, but not after placebo (Figure 1). ADMA and SDMA decreased slightly after LPS and after placebo administration, but this decrease was not significant (Table 2). The L-arginine/ADMA ratio declined significantly after LPS by a median of 25 (CI: 13, 68; P < 0.02), but not in subjects exposed to placebo. Compared with controls, the L-arginine plasma levels and the L-arginine/ADMA ratio were lower in the LPS treated subjects 3.5 h after LPS (both P < 0.05).

**DISCUSSION**

Our previous studies have consistently shown that the maximum haemodynamic effects of LPS, such as systemic vasodilation, endothelial dysfunction and adrenoceptor hyporeactivity, occur approx. 4 h after administration [18]. The increase in body temperature and leucocytosis was paralleled by a significant decrease of L-arginine and the L-arginine/ADMA ratio in plasma collected at equivalent time points. Since ADMA competes with L-arginine for transmembranous transport [22] and binding to NOS [1], a relative deficiency in L-arginine could lead to altered NO generation. Consequently, a decreased L-arginine/ADMA ratio is thought to result in endothelial dysfunction [23]. In contrast, an elevation of the L-arginine/ADMA ratio enhances systemic NO production [24]. This suggests that a deficiency of L-arginine, as found in the present experiments, could affect vascular tone and endothelium-dependent vasodilation.

Our results are in accordance with a recent work which demonstrated that LPS administration to rats results in a significant reduction of L-arginine plasma concentrations [25]. However, ADMA decreased and SDMA increased during endotoxaemia in this study [25]. This discrepancy might be due to species differences or to the considerably higher doses of LPS (8 mg/kg of body mass) administered to the rats.

The mechanisms that cause reduced concentrations of L-arginine after LPS were not revealed by our findings. Enhanced renal excretion is unlikely to account for our results, since L-arginine is excreted in negligible amounts by the human kidney. We have recently demonstrated consumption of antioxidants after LPS [26].
Supplementation with high doses of vitamin C was able to restore endothelial dysfunction in LPS challenged volunteers. In agreement with these experiments, L-arginine concentrations were shown to be significantly consumed in paediatric patients with sepsis as a result of increased oxidation [27]. L-Arginine oxidation was not measured in our present study. We can therefore only speculate that oxidation of L-arginine has occurred. Alternatively, LPS was shown to increase arginase activity in alveolar macrophages [28], which metabolize L-arginine. This mechanism has not been demonstrated in humans to date. Also, expression of iNOS (inducible isoform of NOS) with increased NO production [29–31] in alveolar macrophages [28], which metabolize L-arginine. This mechanism has not been demonstrated in humans to date. Also, expression of iNOS (inducible isoform of NOS) with increased NO production [29–31] could have reduced circulating L-arginine concentrations. However, the latter is rather unlikely, as acute vascular hyporeactivity was detectable in the human endotoxin model in the absence of iNOS expression or altered NO bioactivity [18]. Injection of LPS in rats increases the expression of cationic amino acid transporters [32], which is another possible mechanism for decreased circulating L-arginine levels.

Acutely elevated levels of endotoxin and acute systemic inflammation are associated with increased risk of myocardial infarction [33,34]. The cause for this finding could be dysfunctional endothelium during acute inflammatory states [34]. ADMA production has been increased in human umbilical vein endothelial cell cultures by prolonged exposure to tumour necrosis factor-α, probably via reduced activity of the enzyme dimethylarginine dimethylaminohydrolase [35]. However, these experiments cannot be extrapolated to the early changes during endotoxin-induced inflammation in our present study. Elevated inflammatory cytokines have only been detected during the first 3 h after LPS administration in humans [36,37]. Consequently, the effects of continuously increased cytokines on ADMA are not accessible in the human endotoxin model. Thus, the mechanisms behind endothelial dysfunction after LPS administration might be different than those seen in chronic inflammation.

All subjects were studied under fasting conditions. Confounding effects of different L-arginine intake with food are therefore unlikely. Daytime variations of L-arginine were small in controls and therefore of little influence on our results.

In conclusion, E. coli endotoxin-induced acute systemic inflammation reduces plasma concentrations of L-arginine and decreases the L-arginine/ADMA ratio in healthy subjects. This could contribute to vascular impairment, which is observed during endotoxin-induced experimental inflammation in humans.

Table 2  Plasma concentrations of L-arginine, ADMA and SDMA, and the L-arginine/ADMA ratio, before and after LPS (20 units/kg of body mass; n = 8) or placebo (n = 9) administration

<table>
<thead>
<tr>
<th></th>
<th>Baseline</th>
<th>3.5 h</th>
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<tbody>
<tr>
<td>L-Arginine (µmol/l)</td>
<td></td>
<td></td>
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<tr>
<td>LPS</td>
<td>66 (56, 88)</td>
<td>48 (40, 60)†</td>
</tr>
<tr>
<td>Controls</td>
<td>79 (71, 91)</td>
<td>69 (58, 88)</td>
</tr>
<tr>
<td>ADMA (µmol/l)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LPS</td>
<td>0.46 (0.37, 0.52)</td>
<td>0.40 (0.37, 0.44)</td>
</tr>
<tr>
<td>Controls</td>
<td>0.50 (0.43, 0.58)</td>
<td>0.45 (0.38, 0.56)</td>
</tr>
<tr>
<td>SDMA (µmol/l)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LPS</td>
<td>0.45 (0.34, 0.51)</td>
<td>0.40 (0.33, 0.46)</td>
</tr>
<tr>
<td>Controls</td>
<td>0.46 (0.41, 0.51)</td>
<td>0.44 (0.38, 0.56)</td>
</tr>
<tr>
<td>L-Arginine/ADMA ratio</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LPS</td>
<td>159 (137, 193)</td>
<td>135 (103, 146)†</td>
</tr>
<tr>
<td>Controls</td>
<td>166 (136, 195)</td>
<td>154 (125, 191)</td>
</tr>
</tbody>
</table>

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