Nitrotyrosine formation, iNOS and the Na\(^+\)/K\(^+\)-ATPase activities in sepsis: The possible effects of CAPE

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Abstract

Sepsis is a response to infection characterized by the formation of highly reactive oxygen and nitrogen substances. The rat kidney was chosen for this purpose because many important inflammatory mediators, including inducible nitric oxide synthase (iNOS) and nitrotyrosine (nTyr) production, are expressed by kidney cells following either lipopolysaccharide (LPS) or bacterial challenge. The present study was aimed at investigating the relationship between nTyr formation with iNOS and Na\(^+\)/K\(^+\)-ATPase activities. We were also aimed at investigating the possible role of caffeic acid phenethyl ester (CAPE) on endogenous nTyr production, Na\(^+\),K\(^+\)-ATPase and iNOS activities in the kidney. Kidney Na\(^+\)/K\(^+\)-ATPase activity were maximally inhibited 6h after LPS injection and LPS treatment significantly increased iNOS activity of kidney. The regression analysis displays negative correlation between Na\(^+\)/K\(^+\)-ATPase activity and nTyr levels of LPS treated animals. Na\(^+\)/K\(^+\)-ATPase activity were also negatively correlated with iNOS activity in LPS–treated rats. These data suggest that nitric oxide (NO\(^\cdot\)) and peroxynitrite (ONOO\(^-\)) contribute to the development of oxidant injury. Furthermore, the source of NO\(^\cdot\) may be iNOS. iNOS are expressed by the kindey, and their activity may increase following LPS administration. Also, NO\(^\cdot\) and ONOO\(^-\) formation inhibited Na\(^+\)/K\(^+\)-ATPase activity. This results also have strongly suggested that bacterial LPS disturbs activity of membran Na\(^+\)/K\(^+\)-ATPase that may be an important component leading to the pathological consequences such as renal dysfunction in which the production of reactive nitrogen substance (RNS) are increased as in the case of LPS challenge. CAPE treatment was decreased nTyr production and iNOS activities and increased Na\(^+\)/K\(^+\)-ATPase activity. These data suggest that CAPE treatment contribute to the decrease of oxidant injury.

Key words: Nitrotyrosine, Na\(^+\)/K\(^+\)-ATPase, iNOS, kidney, CAPE

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Introduction
The expression of inducible nitric oxide synthase (iNOS) protein is induced by lipopolysaccharide (LPS) in many cells and tissues including kidney. In other words, iNOS and cytokine production are up-regulated by LPS in the kidney (1, 2). Reduction in glomerular filtration rate (GFR) and hypotension is associated with up-regulation of iNOS in LPS–induced septic animals (1, 3). Inner medullary collecting duct cells and renal proximal tubule can produce NO via expression of an iNOS isoform (1, 4).

NO and ONOO• contribute to the development of oxidant injury. The cell types responsible for NO and superoxide generation in the kidney in response to LPS are not known. Interestingly, proximal tubule constitutive NOS and iNOS are both capable of generating superoxide in addition to NO (1, 5). The production of both NO and superoxide increases in septic shock. The cogeneration of these molecules is known to yield ONOO•, which preferentially nitrates tyrosine residues in protein and non–protein orgins (1, 6). The production of nitrotyrosine (nTyr) in the kidney has been associated with several pathological conditions (1, 7).

\[ \text{Na}^+\text{/K}^+\text{-ATPase}\]

is an energy utilizing transmembrane enzyme. It is responsible for the maintenance of ionic gradients of Na+ and K+. Na+/K+–ATPase has been shown to be very susceptible to free radicals and membrane lipid peroxidation (1, 8). It has been reported that NO derived products (NO• and ONOO•) inhibit Na+/K+–ATPase activity via the possible oxidation of thiol groups of the enzyme in many cells and tissues (1, 9-13). Previous studies have been demonstrated that ONOO• signaling participates in the regulation of renal Na+/K+–ATPase activity (1, 12). Furthermore, it has been demonstrated that the endogenous NO plays a direct inhibitory effect over Na+/K+–ATPase activity in the kidney (1, 14). It has been also reported that NO• generated by iNOS inhibits Na+/K+–ATPase activity in an autocrine fashion (1,15).

Caffeic acid phenethyl ester (CAPE), an active component of propolis from honeybee hives, is known to have anticarcinogenic, immunomodulatory, antiinflammatory, and antioxidant properties. It has been demonstrated that CAPE is an agent which is a free radical scavenger and activates antioxidant enzymes (16).

The aim of this study is to evaluate the effects of CAPE on nTyr formation, Na+/K+–ATPase and iNOS activities in septic kidney.

Method
Rats (250–300g) were divided into 3 groups (n=10 each group). Group 1 animals were intraperitoneally injected with saline (control group). Group 2 animals were intraperitoneally injected with LPS, 20 mg/kg single dose (LPS–treated group) (17). Group 3 animals were intraperitoneally injected with CAPE, 10 µmol/kg single dose (CAPE–treated group) following a 20 mg/kg single dose of LPS injection (18-24).

Animals were sacrificed under ketamin/xylazin (60-10 mg/kg i.p single dose) anesthesia at 6h after injections (1, 25, 26). After sacrifice, the kidneys were removed, washed with cold NaCl 0.9% and immediately kept frozen in liquid nitrogen. The kidney tissues were stored at -70°C until use.

Measurement of 3-nitrotyrosine
Tissue sample was homogenized in buffer
(50mM potassium-phosphate buffer, pH:7.4) and hydrolysed in 6 N HCl at 90–110°C for 18-24 h. Hydrolyzed samples were centrifuged at 3000 rpm for 10 min, the supernatants were separated for the analysis of nTyr levels. The samples were analyzed on a Agilent 1200 diode array detector HPLC apparatus. The analytical column was 5μm pore size Spherisorb ODS–2 C18 reverse–phase column (4.6×250mm, Alltech, Dearfield, IL, USA). The guard column was a C18 cartridge (Alltech, Dearfield, IL, USA). The mobile phase was 50 mmol/L sodium acetate/50mmol/L citrate/8% (v/v) methanol pH 3.1. HPLC analysis was performed under isocratic conditions at a flow rate of 1ml/min and UV detector set at 274nm. Concentrations of nTyr were calculated from nTyr standard curve and expressed as nmol/g tissue (1, 11, 25, 27, 28).

Measurement of Na⁺/K⁺-ATPase activity
Tissue homogenate was prepared for the Na⁺/K⁺-ATPase study using a glass–homogenizer. Homogenates were centrifuged at 3000 rpm for 5 min and supernatant was separated. Na⁺/K⁺-ATPase activity in the supernatant was determined. Na⁺/K⁺-ATPase activity was assessed by the measurement of the produced inorganic phosphate and results were expressed as spesific activity (μmol P[ sub i ] / h / mg protein) (1, 11, 25, 28, 29).

Assay for nitric oxide synthase activity
Tissues were homogenized with a buffer containing 10mM HEPES, 0.32M sucrose, 0.1mM EDTA, 1mM dithiothreitol, 10μg of soyabean tripsin inhibitör/ml, 10μg of leupeptin/ml, 2μg of aprtinin/ml and 1mg of PMSF/ml, adjusted to pH 7.4. The homogenates were then centrifuged at 100000×g for 1h. NO synthesis was measured by a previously described method (1, 30, 31), in which the oxidation of oxyhaemoglobin to methaemoglobin by NO is monitored spectrophotometrically. The absorption difference between 401 and 411 nm was continuously monitored with a dual–wavelength recording spectrophotometer by using a bandwith of 2 nm, at 37°C.

Statistical calculations
The data resulting from each group were expressed as the mean ± S.E.M. A Mann Whitney U test t-test was used to compare means between the two groups using SPSS 10.0. Linear regression analysis was applied where indicated. A p value < 0.05 was considered significant.

Results
Nitrotyrosine levels, iNOS and Na⁺/K⁺-ATPase activities were shown in Table 1. In our study, nTyr levels have been hardly detected in control rat kidneys but nTyr levels have been detected markedly in kidneys of septic animals. nTyr levels were also significantly increased in the LPS-treated group when compared to the control (P< 0.05) (Table 1).

<table>
<thead>
<tr>
<th></th>
<th>Control (n=10)</th>
<th>LPS–treatment (n=10)</th>
<th>CAPE–treatment (n=10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>iNOS (nmol/min/g tissue)</td>
<td>0.375 ± 0.044</td>
<td>0.865 ± 0.118</td>
<td>0.510 ± 0.069</td>
</tr>
<tr>
<td>nTyr (nmol/g tissue)</td>
<td>Hardly detectable</td>
<td>4.633 ± 0.716</td>
<td>0.712 ± 0.111</td>
</tr>
<tr>
<td>Na⁺K+ATPase (μmol P[ sub i ]/h/mg protein)</td>
<td>2.690 ± 0.172</td>
<td>1.352 ± 0.158</td>
<td>2.765 ± 0.065</td>
</tr>
</tbody>
</table>

CAPE: Caffeic acid phenethyl ester, iNOS: inducible nitric oxide synthase, LPS: lipopolysaccharide, nTyr: 3-nitrotyrosin.
The regression analysis between Na\(^{+}\)/K\(^{+}\)-ATPase activity and nTyr levels of LPS–treated animals revealed negative correlation (Figure 1). Similarly, Na\(^{+}\)/K\(^{+}\)-ATPase and iNOS activities were also negatively correlated (Figure 2).

![Figure 1: Negative correlation between Na\(^{+}\)/K\(^{+}\)-ATPase activity and nTyr formation in kidney of septic rats.](image1)

![Figure 2: Negative correlation between Na\(^{+}\)/K\(^{+}\)-ATPase activity and iNOS measurements.](image2)

In our this study, Na\(^{+}\)/K\(^{+}\)-ATPase activity significantly decreased in LPS–treated animals compared to control animals (P< 0.05) (Figure 3). Although iNOS activities were low, even barely detectable in control animals, iNOS activity in LPS-treated groups were significantly increased (P< 0.05) (Figure 4).

In this study, Na\(^{+}\)/K\(^{+}\)-ATPase activity significantly increased in CAPE–treated rat kidneys compared to LPS animals (P< 0.05) (Figure 3). Although iNOS activity and nTyr formation were high level detectable in kidney of LPS–treated animals, these parameters were significantly decreased in CAPE–treated animals (P< 0.05) (Table 1).
Discussion
In our study, there is a significant increase in iNOS activity in kidney following 6h after injection of LPS to rats. This findings is in accord with previous observations in liver (1,25), and also consistent with those of others showing that NO$^-$ or its metabolites are significantly increased in many organs after LPS administration (1, 32-34).

It is demonstrated that iNOS mRNA induced by LPS (1, 32) and it has been reported that LPS–treatment caused increase in the amount of iNOS mRNA in many tissues (1, 35). In addition, Mayeux et al. report that proximal tubules express a calcium/calmodulin–dependent NOS activity that is increased in vivo by LPS (1, 36). Our results are also in accordance with those of previous studies on increased iNOS level in sepsis.

In this study, we indicated that excess formation of reactive nitrogen substance (RNS) is responsible for the impaired Na$^+$/K$^+$-ATPase activity in septic kidney. Guzman et al reported that NO$^-$ generated by mause proximal tubule epithelial cell iNOS inhibits Na$^+$/K$^+$-ATPase activity (1, 15). Our results are in accordance with those of previous studies on impaired Na$^+$/K$^+$-ATPase activity in septic animals.

A negative correlation observed between
Na<sup>+</sup>/K<sup>+</sup>-ATPase and iNOS activity as well as nTyr levels in septic animals strengthens the direct involvement of RNS. These data suggest that ONOO⁻ signaling participates in the regulation of renal Na<sup>+</sup>/K<sup>+</sup>-ATPase activity and the endogenous NO⁻ formation due to elevated iNOS activity, plays a inhibitory role on Na<sup>+</sup>/K<sup>+</sup>-ATPase activity in the kidney. Furthermore, this finding is also in accord with previous observations in kidney (1, 12, 14). Depletion of glutathione and other protective antioxidants by RNS may greatly contribute to increasing amount of reactive species, which may also account for impaired activity of Na<sup>+</sup>/K<sup>+</sup>-ATPase (1, 37). ONOO⁻ has been shown to directly oxidize a SH group of the active site of the Na<sup>+</sup>/K<sup>+</sup>-ATPase (1, 9).

Song YS et al. reported that CAPE inhibits nitric oxide synthase gene expression and enzyme activity (38). Çelik S and Erdoğan S. demonstrated that CAPE protects brain against oxidative stress and inflammation induced by diabetes in rats (39). Kassim M. et al reported that CAPE is scavenger of peroxynitrite in vitro and in sepsis models (40). Çakır T et al. showed that CAPE prevents methotrexate-induced hepatorenal oxidative injury in rats (41). Our findings are in accordance with those of previous studies on antioxidant and protective effects of CAPE.

In summary, our results show that although nTyr levels and iNOS activity were increased, Na<sup>+</sup>/K<sup>+</sup>-ATPase activities were decreased in rat kidney exposed to LPS. Thus, the negative correlation of Na<sup>+</sup>/K<sup>+</sup>-ATPase activity was observed with both iNOS activity and nTyr levels in the kidney treated LPS. In conclusion, the present study have indicated that both endogenous production of NO⁻ via iNOS activity and simultaneously superoxide generation are stimulated in response to LPS. Thus, NO⁻ and superoxide react spontaneously to form nitrating agent and versatile oxidant ONOO⁻. Therefore, although Na<sup>+</sup>/K<sup>+</sup>-ATPase activity is impaired, iNOS activity is increased in response to LPS. In this sence, our this study have demonstrated that RNS-dependent kidney dysfunction also include the modification in membrane Na<sup>+</sup>/K<sup>+</sup>-ATPase, which impairs the activity of the enzyme. This event may be a crucial component leading to pathological consequences such as kidney dysfunction in which the production of RNS are increased as in the case of LPS challenge. In addition, our results also have suggested that CAPE treatment decreased kidney tissue injury in sepsis.

Conflicting Interest
The authors have no competing interests

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