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Impaired Na^+,K^+-ATPase activity as a mechanism of reactive nitrogen species-induced cytotoxicity in guinea pig liver exposed to lipopolysaccharides

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Abstract

In animal models of endotoxin, the excess production of NO and the reactive nitrogen species (RNS), are potent oxidant and nitrating agents, lead to lipid peroxidation, apoptosis, tissue dysfunction and injury and inactivate enzymes in many cell types. Although liver functions are well known to deteriorate following bacterial infection, the underlying specific mechanism(s) remain a matter of considerable debate. Therefore, the aim of the present study was to determine the *in vivo* effect of bacterial lipopolysaccharides (LPS) on Na^+,K^+-ATPase activity of guinea pig liver, and to investigate the possible contribution of RNS by measuring of iNOS activity and 3-nitrotyrosine (nTyr) levels. Liver Na^+,K^+-ATPase activity were maximally inhibited 6 h after LPS injection ($p < 0.001$). nTyr was not detectable in liver of normal control animals, but was detected markedly in LPS exposed animals. LPS treatment significantly increased iNOS activity of liver ($p < 0.001$). The regression analysis revealed a very close correlation between Na^+,K^+-ATPase activity and nTyr levels of LPS treated animals ($r = –0.863$, $p < 0.001$). Na^+, K^+-ATPase activity were also negatively correlated with iNOS activity ($r = –0.823$, $p < 0.003$) in inflamed tissues. Our results have strongly suggested that bacterial LPS disturbs activity of membrane Na^+,K^+-ATPase that may be an important component leading to the pathological consequences such as hepatocyte cell loss and dysfunction in which the production of RNS are increased as in the case of LPS challenge. (Mol Cell Biochem **259**: 53–57, 2004)

Key words: 3-nitrotyrosine, Na^+,K^+-ATPase, iNOS, liver, guinea pig

Introduction

Sepsis is a response to infection characterized by the production of inflammatory mediators and cytokines, such as TNF-α, IL-1β, IFN-γ, CD14, ICAM-1 [1, 2]. Highly reactive oxygen and nitrogen species (RNS), including nitric oxide (NO), nitronium ions, nitrogen dioxide radical, nitroxyl anion, peroxynitrite, and peroxynitrous acid has also been shown to produce in many cell types following lipopolysaccharide (LPS) challenge [3–5]. NO although may protect the liver under some circumstances, but also has the potential to promote injury [3, 5–7]. The inducible form of nitric oxide synthase (iNOS) is importantly involved in inflammatory responses and is produced in inflamed liver, resident cells, including activated hepatocytes and Kupffer cells, as well as infiltrating polymorphonuclear leucocytes and monocytes [8–10]. Previous studies showed that iNOS-derived NO regulates proinflammatory genes *in vivo*, thereby contributing to inflammatory liver injury by stimulating of TNF-α production [11]. Excess NO is also a reason for inhibited hepatic mitochondrial energy metabolism and ATP depletion [12].

The formation of RNS depends, in part, on the reaction of

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NO with intracellular oxygen and superoxide that results in the formation of peroxynitrite [13, 14]. Especially peroxynitrite and other RNS are potent oxidant and nitrating agents that lead to lipid peroxidation, depletion of antioxidant defense, inactivation of enzymes, increased apoptosis of many cell types [13–15]. RNS has been shown to affect cell-resting membrane potential to impair active cation uptake by sarcoplasmic reticulum [16, 17], and to alter integrity and permeability of cellular membranes [5]. Functional and morphological changes in liver observed after chronic exposure to RNS has been shown to the results of persistent mitochondrial and nuclear DNA damage [6, 7]. In this respect, the studies highlight the importance of inhibition of NO/$ONOO^-$ pathway in preventing RNS-mediated tissue injury in lipopolysaccharide (LPS)-induced endotoxemia [17, 18]. On the other hand, RNS-induced nitration of tyrosine residues leads to formation of 3-nitrotyrozine (nTyr), which has been used as a biomarker for the production of RNS [19, 21]. Previous studies have demonstrated that the treatment of membranes with peroxynitrite or LPS results in oxidative damage as evidenced by the presence of nitration of tyrosine residues; this may alter the function of membrane enzymes [21, 22].

Na^+,K^+-ATPase is an energy utilizing transmembrane enzyme, which is responsible for the maintenance of ionic gradients of Na^+ and K^+ ions across the cell membrane. The activity of Na^+,K^+-ATPase has been shown to very susceptible to free radicals and membrane lipid peroxidation [23]. Previously, it has been demonstrated that NO-derived products ($^{\cdot}NO_2$ and $ONOO^-$) or SNAP (a NO donor) or SIN-1 (a peroxynitrite donor) inhibit Na^+,K^+-ATPase activity via the possible oxidation of thiol groups of the enzyme, leading to a decrease in the activity of the enzyme in cerebral cortex [24, 25], erythrocytes [21], kidney proximal tubule [22] and liver plasma membrane [26].

Although hepatocyte functions are well known to deteriorate following bacterial infection, the underlying specific mechanism(s) responsible for LPS-induced changes remain a matter of considerable debate. In the light of above-mentioned interactions, our study was undertaken to determine the effects of LPS on Na^+,K^+-ATPase activity of guinea pig liver, and to investigate the possible role of RNS. The transient natures of the species make it difficult to quantify the production of RNS *in vivo*. The assay of nTyr in protein has been proposed as an indirect marker of RNS production [27]. We also measured iNOS activity in liver to elucidate underlying mechanisms.

Materials and methods

All the reagents and chemicals used in these experiments were of analytical grade of highest purity. All organic solvents were HPLC grade. Our chemicals were obtained from Sigma, Merck Company or Boehringer Mannheim.

Guinea pigs (200–400 g) were divided into 2 groups (n = 10 for each group) and were injected intraperitoneally with *Escherichia coli* (*E. coli*) dosed at 12×10^9 colony-forming units/kg. Livers were collected 6 h after administration of *E. coli* [4, 21]. Livers were removed, washed with cold NaCl 0.9% and immediately placed in liquid nitrogen. The liver tissues were frozen at –70°C until use.

Measurement of Na^+, K^+-ATPase activity

10% liver 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 specific activity (µmol Pi/h/mg protein) [21, 28]. All experiments were performed in duplicate.

Measurement of tyrosine nitration

0.5 g liver in 1.5 ml buffer (50 mM KPO_4; pH 7.4) was homogenized, adjusted to ~4 mg protein/ml and hydrolyzed in 6 N HCl at 100°C for 18–24 h. After centrifugation at 3000 rpm for 10 min, the supernatants were separated for the measurement of the 3-nitrotyrosine levels. The samples were analyzed on a Hewlett Packard 1050 diode array detector HPLC apparatus (Hewlett Packard, Waldbron, Germany). The analytical column was 5 µm pore size Spherisorb ODS-2 C_{18} reverse-phase column (4.6 × 250 mm; Alltech, Dearfield, IL, USA). The guard column was a C_{18} cartridge (Alltech). The mobile phase was 50 mmol/l sodium acetate/50 mmol/l citrate/8% (v/v) methanol pH 3.1. HPLC analysis was performed under isocratic conditions at a flow rate of 1 ml/dk and UV detector was set at 274 nm. nTyr peaks were determined according to its retention time and confirmed by spiking with added exogenous 3nTyr. Concentrations of nTyr were calculated from nTyr standard curve and expressed as nmol/g tissue [21].

Assay for nitric oxide synthase

The livers were homogenized with 5 vol. of a buffer containing 10 mM-Hepes, 0.32 M sucrose, 0.1 mM EDTA, 1 mM dithiothretiol, 10 µg of soyabean tripsin inhibitor/ml, 10 µg of leupeptin/ml, 2 µg of aprotinin/ml and 1 mg of phenylmethanesulphonyl fluoride/ml, adjusted to pH 7.4 (at room temperature) with NaOH. The homogenates were then centrifuged at 100,000 × g for 1 h. NO synthesis was measured by a previously described method [29], in which the oxidation of oxyhaemoglobin to methaemoglobin by NO is moni-

Table 1. iNOS activity, 3-nitrotyrosine level and Na^+,K^+-ATPase activity in liver homogenates of control or exposed guinea pigs with *E. coli* lipopolysaccharides

	iNOS (nmol/min/g tissue)	3-nitrotyrosine (nmol/g tissue)	Na^+,K^+-ATPase (μmol Pi/h/mg protein)
Control (n = 10)	0.746 ± 0.063	not detectable	0.393 ± 0.032
LPS treatment (n = 10)	1.278 ± 0.027	3.941 ± 0.351	0.203 ± 0.013

Data are means ± S.E.M.

tored spectrophotometrically. The absorption difference between 401 and 411 nm was continuously monitored with a dual-wavelength recording spectrophotometer by using a bandwidth of 2 nm, at 37°C.

Statistical calculations

The data resulting from each experimental group were expressed as the mean ± S.E.M. An unpaired Student's *t*-test was used to compare means between the two groups. Linear regression analysis was applied where indicated. A p value < 0.05 was considered significant.

Results

nTyr levels, iNOS and Na^+,K^+-ATPase activities in liver of control and LPS injected animals are given in Table 1.

nTyr was not detectable in liver homogenates of normal control animals. As can be seen in Table 1, nTyr levels were detected markedly in hepatocytes when the animals were exposed to LPS.

iNOS activity was low level detectable in liver of control rats. LPS treatment significantly increased iNOS activity in liver ($p < 0.001$). In fact, the liver has also been shown to contain a low constitutive activity of NO synthase [30].

Liver Na^+,K^+-ATPase activity were inhibited about 50% after LPS injection ($p < 0.001$). The regression analysis revealed a very close correlation between Na^+, K^+-ATPase activity and nTyr levels of LPS treated animals ($r = –0.863$, $p < 0.001$) (Fig. 1). Na^+, K^+-ATPase activity were also correlated negatively with iNOS activity ($r = –0.823$, $p < 0.003$) (Fig. 2).

Discussion

Bacterial infections are often complicated by hepatic dysfunction in humans and animals. Despite extensive efforts, the mechanisms responsible for this complication remain poorly understood. Tumor necrosis factor, reactive oxygen and nitrogen species, and NO, have been demonstrated as mediators for systemic inflammatory response, hepatic injury and dysfunction [1–5, 27]. A number of investigations have confirmed that NO is generated in excessive concentrations as a consequence of iNOS stimulation by LPS or cytokines leading to inhibition in hepatocyte mitochondrial energy metabolism, apoptosis and DNA damage [6, 7, 10]. It is also believed that NO itself or highly reactive and cytotoxic RNS formed from NO and superoxide anion account for cellular injury and dysfunction associated with LPS-induced liver inflammation [3, 5, 7, 8, 13, 27]. In the mechanism(s) of LPS-induced cellular degeneration, the increased production of RNS is put forwarded as triggered factors [5, 7].

In this study, we have found that there is a significant increase in iNOS activity and nTyr levels in liver following 6

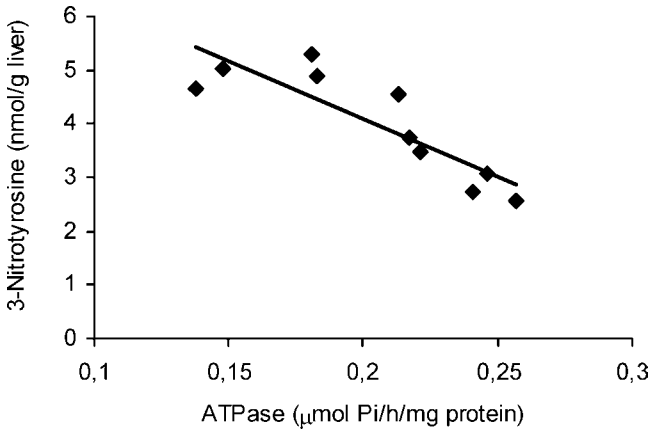

Fig. 1. Correlation between Na^+,K^+-ATPase activity values and 3-nitrotyrosine concentrations in liver of guinea pigs exposed to bacterial lipopolysaccharide ($r = –0.863$, $p < 0.001$).

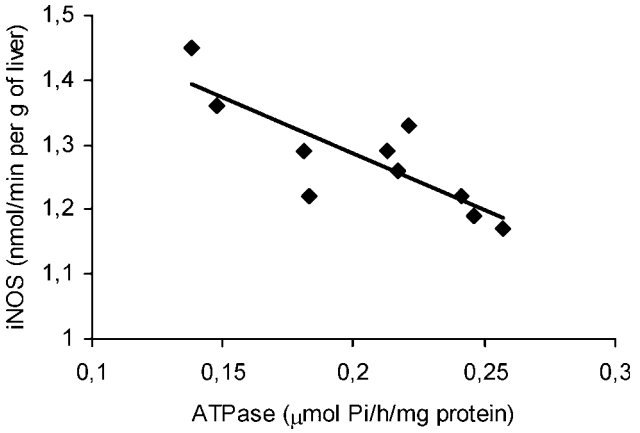

Fig. 2. Correlation between Na^+,K^+-ATPase activity values and iNOS measurements in liver of guinea pigs exposed to bacterial lipopolysaccharide ($r = –0.823$, $p < 0.003$).

h after administration of *E. coli* LPS to the animals. This finding is in accord with our previous observations in plasma [4] and erythrocytes [21], and also consistent with those of others showing that NO or its metabolites are significantly increased in many organs as well as liver after LPS administration [5, 10, 31]. The liver NOS has been shown to mainly present in the parenchymal-cell fraction and is induced by endotoxin maximally at 6 h after injection [5]. Increased nitration of tyrosine residues was demonstrated in hepatocyte membrane in response to peroxynitrite-induced oxidative stress by LPS [5, 7, 32]. Peroxynitrite is formed at almost diffusion-limited rates by the reaction between NO and superoxide ($\cdot O_2^-$), and rapidly attack phenolic targets, yielding stable and unique products. Peroxynitrite reaction with tyrosine gives 3-nitrotyrozine (3-NO_2-Tyr) in high yield [33]. Because nTyr is a stable end product of peroxynitrite oxidation, assessment of its plasma or tissue concentrations is considered as a useful marker of peroxynitrite or other RNS-dependent damage *in vivo* [27]. The most attempts to detect nTyr used a combination of high-performance liquid chromatography with ultraviolet or electrochemical detection, or immunochemical methods [34, 35]. UV detection is sufficiently sensitive for determination of nTyr *in vitro* [21, 34, 35] but has been reported generally not sensitive enough for quantitation of nTyr *in vivo* [33]. Although HPLC with electrochemical detection is emerging as a technique of choice for sensitive, selective, and facile instrumental determination of nTyr [33, 35], we and other investigators used UV detection for nTyr analysis in different biological samples successfully [4, 21, 36, 37]. As expected, in the present experiments we could not able to detect nTyr in normal rat liver, but the nTyr levels was determined at micro mol/liter levels by UV detection in LPS-treated rat hepatocytes. The nTyr levels of normal animals would be below the limit of detection of the HPLC-UV analysis capacity. Although nTyr has been reported to generally not detectable in the plasma or some tissues of healthy subjects, electrochemical detection is known to at least 100 times more sensitive than UV detection [34].

Reactive nitrogen species-induced alterations in organ architecture were shown to associate with organ dysmetabolism or the modification of biochemical markers of organ function, such as enzyme activity [5, 6–12, 21]. In the present study, we demonstrated, for the first time, that excess production of RNS is responsible for the impaired Na^+,K^+-ATPase activity in inflamed guinea pig liver. The negative correlation observed between Na^+,K^+-ATPase and iNOS activity as well as nTyr levels in LPS treated animals strengthens the direct involvement of RNS. This finding confirms previous observations showing that LPS is a hepato-degenerative factor due to increased expression of iNOS [7, 11], and the liver plasma membrane Na^+,K^+-ATPase activity is markedly inhibited by a NO donor or a peroxynitrite donor [26]. The results of our current study is not parallel totally with the results of our previous study, showing that Na^+,K^+-ATPase activity of erythrocytes is significantly inhibited when they exposed to peroxynitrite *in vitro*, but unchanged in *E. coli* injected rat [21]. This discrepancy may be explained by the difference between the studied tissues and their intracellular antioxidant capacity. Because, it has been demonstrated that the increased erythrocyte antioxidant capacity plays an important protective role against oxidative effects of peroxynitrite induced by E. coli LPS [21]. The activity of the transmembrane enzyme Na^+,K^+-ATPase is very susceptible to free radicals and membrane lipid peroxidation [23, 25]. Lipid peroxidation has been shown to alter Na^+,K^+-ATPase function by modification at specific active sites in a selective manner [25]. 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^+,K^+-ATPase in liver [7]. Peroxynitrite has been shown to directly oxidize a SH group of the active site of the Na^+,K^+-ATPase and then inhibits this enzyme activity in porcine cerebral cortex [24]. It has been shown that exposure of peroxynitrite not only results in impairment in hepatocyte membrane integrity and Na^+,K^+-ATPase, but also initiates a cascade of events that rapidly leads to damage of both nuclear and mitochondrial DNA in human hepatocytes [7]. Peroxynitrite inhibits mitochondrial energy metabolism in multiple steps, and disrupts ATP formation, which was suggested as a common mechanisms of hepatocellular injury induced by various oxidant chemicals [12].

In conclusion, the present study have demonstrated that RNS-dependent hepatocyte abnormalities also include the modification in membrane Na^+,K^+-ATPase, which impairs the activity of the enzyme. This event may be a crucial component leading to pathological consequences such as hepatocyte cell loss and dysfunction in which the production of RNS/ROS are increased as in the case of LPS challenge.

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