Inhibition of Nitrotyrosine Formation Reduces Endotoxin-Induced Liver Injury Irrespective of TNF-α

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Summary  The hypotheses that nitrotyrosine formation is a downstream mechanism following TNF-α production in lipopolysaccharide (LPS)-induced liver injury and its inhibition reduces the injury were examined. Under anesthesia, experiments were performed 6 h after the intravenous administration of LPS (10 mg/kg) or saline using male Sprague-Dawley rats (300–400 g). Liver injury was evaluated in 4 groups: control (n=4), LPS alone (n=5), LPS + quercetin (n=5), LPS + clodronate (n=5) by plasma alanine aminotransferase (ALT) level, nitrotyrosine concentration in liver and histological changes. Quercetin, an inhibitor of nitrotyrosine formation was injected at a dose of 50 mg/kg i.p. 24 h before the LPS injection and clodronate, a Kupffer cell remover was injected i.v. at a dose of 0.2 ml (0.23 M) 24 h before the LPS injection. Nitrotyrosine was measured by enzyme-linked immunosorbent assay and high performance liquid chromatography-electrochemical detection methods. In the LPS group, the liver contained 1,403 ± 240 ng nitrotyrosine/g protein (control: 21.2 ± 1.7 ng/g) and plasma ALT was elevated (1,781 ± 455 IU/liter vs. control 55 ± 5 IU/liter). Nitrotyrosine formation was decreased in the LPS + quercetin and LPS + clodronate groups (240.6 ± 47.6 and 301.4 ± 26.2 ng/g protein, respectively) and the increase of plasma ALT was attenuated (486 ± 20.7 and 79.8 ± 9.4 IU/liter, respectively). The plasma TNF-α level was markedly elevated in the LPS group (1,124.1 ± 67.6 pg/ml). It remained high in quercetin groups (1,292.4 ± 159.9 pg/ml) but was very low in the clodronate group (34.8 ± 5.3 pg/ml). LPS-induced liver injury involves nitrotyrosine formation and treatments to decrease its formation attenuated the injury. The protection was observed without affecting TNF-α level; suggesting that the nitrotyrosine formation is a downstream mechanism for the injury following TNF-α production.

Key Words: nitrotyrosine, endotoxin-induced liver injury, TNF-α, quercetin, clodronate, Kupffer cells

Introduction

In septic shock, a complex cascade of inflammatory mediators results in severe liver injury. Endotoxin from Gram-negative bacteria, lipopolysaccharide (LPS) is often a primary stimulus for the cascade, inducing secretion of proinflammatory cytokines such as TNF-α and interleukin (IL)-1β by monocytes/macrophages [1–3]. It was therefore hoped that TNF-α-antibody and/or IL-1β receptor blockers would ameliorate septic shock, but studies in LPS-induced animal models and patients with septic shock gave generally poor results [4–6]. A possible
explanation for the low therapeutic benefit may be that TNF-α and IL-1β also possess anti-inflammatory functions by interacting with the regulatory system of IL-1β receptor antagonist, IL-4, IL-10, and secretory leukocyte protease inhibitor (SLPI) [7]. All of these are anti-inflammatory mediators that attenuate the production of TNF-α and IL-1β in addition to inhibiting the translocation of NF-κB, a transcription factor for many proinflammatory cytokine genes. Therefore, new therapeutic targets should be sought at locations downstream from TNF-α and IL-1β.

Promising therapeutic targets appear to be reactive oxidants such as nitric oxide (NO), superoxide and peroxynitrite, since increased production of these molecules has been demonstrated in endotoxemia [3, 8, 9]. Among reactions by these oxidants the nitration of tyrosine by peroxynitrite is of current interest [10, 11], and nitrotyrosine has been detected in human tissues [12], and in animal models of various diseases [11, 13-15] including the liver of LPS-treated mouse [13, 16]. However, the effect of inhibitor for nitrotyrosine formation on liver damage has not been examined. Furthermore, the causal relationship among TNF-α release, nitrotyrosine formation and liver damage is not known.

Thus, in the present study we tested the hypotheses that inhibition of nitrotyrosine formation would decrease liver damage and the formation of nitrotyrosine may be a downstream reaction induced by TNF-α.

Materials and Methods

Animal model and experimental protocol

Animals were maintained and used according to the National Institute of Health Guidelines for Laboratory Animal Care. The protocol was approved by the Animal Experimentation Committee, School of Medicine, Tokai University.

Sprague-Dawley (SD) rats weighing 300-400 g were used. Rats received an injection of Escherichia coli LPS (0111: B4, Sigma, USA) at a dose of 10 mg/kg body weight (10 mg/ml in bacteriostatic normal saline) or saline, i.v. and designated as LPS and control groups. Rats in the LPS+quercetin group received quercetin (Wako Pure Chemical Ind. Ltd., Osaka, Japan), an inhibitor of nitrotyrosine formation [17] i.p., at a dose of 50 mg/kg body weight 24 h before the LPS injection. Rats in the LPS+clodronate group, were pretreated with clodronate-containing liposomes at a dose of 0.2 ml (0.23 M), i.p. 24 h before the LPS injection. These liposomes were reported to remove more than 90% of the Kupffer cells [1], and we found in a preliminary study that ~90% of Kupffer cells could be depleted. Rats were sacrificed by administering an overdose of pentobarbital sodium at 6 h after the LPS injection. Blood was collected and the liver was excised, snap-frozen in liquid nitrogen and stored at −80°C until use.

Preparation of liposome-encapsulated clodronate

Multilamellar liposomes were prepared by Van Rooijen’s method with slight modification [18]. In brief, 86 mg of phosphatidylcholine (Sigma, USA) and 8 mg of cholesterol (Sigma, USA) were dissolved in chloroform and then sonicated. The liposomes were resuspended in PBS and the stock solution contained 0.23 M clodronate, a level which has been widely used for macrophage depletions [19, 20].

Measurement of nitrotyrosine by HPLC-electrochemical and ELISA methods

Quantitative evaluation of nitrotyrosine in biological materials is usually based on HPLC detection [21] or mass spectrometry [22]. As these techniques have potential methodological pitfalls, such as an unknown artifact peak or low sensitivity [23], we therefore utilized two methods based on different principles.

For HPLC method, each liver was homogenized in 20 mM sodium phosphate, pH 7.4 and the homogenate was centrifuged at 10,000 rpm for 2 min to pellet nuclei and large particles. Then the supernatant was subjected to proteolytic digestion as described by Skinner et al. [24]. For HPLC analysis, we used a dual-channel electrochemical detector (Coulochim II, ESA Inc., USA) set at 600 mV for the first channel and 750 mV for the second channel, as described elsewhere [25]. The identity of the nitrotyrosine peak was confirmed by the retention time, characteristic electrochemical properties and coelution with authentic nitrotyrosine added to each sample. Results were expressed as the percentage of nitrotyrosine to total tyrosine.

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For ELISA method, we used a competitive ELISA introduced by Khan J. et al. [26] with some modification. Briefly the assay was performed in 96-well plates coated with 10 μg/ml nitro-BSA (immobilized antigen) which had been blocked with 1% casein in PBS to prevent non-specific binding. Homogenized liver (50 μl) was added to each well and incubated with immunoaffinity-purified polyclonal anti-nitrotyrosine rabbit IgG (1:500) (100 μl) for 2 h at 37°C. After washing, a peroxidase-conjugated donkey anti-rabbit IgG was added. Color development was initiated by the addition of substrate and terminated by the addition of 2.5 M sulfuric acid. The absorbance was measured at 490 nm with a microplate reader (Spectra Max 250, Molecular Devices, USA). The concentrations of nitrated proteins were estimated from a standard curve, made by nitro-BSA. The results were expressed as ng/g protein assuming nitrotyrosine as nitro-BSA equivalent.

Measurement of plasma levels of nitrate and nitrite
Concentrations of nitrate and nitrite (NOx) in plasma were measured by using Griess regent. Briefly, plasma samples were filtered through a 5,000 NMWL filter (Ultrafree C3, Millipore Ltd., Tokyo, Japan) by centrifugation at 10,000×g for 40 min. The filtered plasma was injected into an automated flow-through spectrophotometric system as described by Green et al. [27] after the reduction of nitrate to nitrite by an on-line cadmium column (JASCO Ltd., Tokyo, Japan).

Measurement of plasma levels of TNF-α
TNF-α protein in plasma was quantified by the use of a rat TNF-α ELISA kit (FactorTest-X, Genzyme, Cambridge, MA, USA), a solid-phase ELISA that employs the multiple antibody sandwich principle. Briefly, a 96-well microplate (pre-coated with monoclonal anti-TNF-α) was used to capture rat TNF-α. After washing, a peroxidase-conjugated polyclonal anti-TNF-α was added and the washing procedure was repeated. A substrate solution was added, which initiated a peroxidase-catalyzed color change that was stopped by acidification. The absorbance was measured at 450 nm using a microplate reader. The calculation was performed by Macintosh computer software.

Statistical analysis
Results were expressed as the mean±SEM. Inter-
group comparisons were made by one-way ANOVA followed by Fisher's protected least significant difference test. A level of \( p < 0.05 \) was considered significant.

**Results**

*Effects of quercetin and clodronate on the liver injury*

The plasma ALT level in the LPS group was 1,781±455 IU/liter (55±5 IU/liter in the control group) but it was significantly reduced in the quercetin and clodronate groups (486.6±20.7 IU/liter; \( p < 0.05 \) and 79.8±9.4 IU/liter; \( p < 0.01 \), respectively) (Fig. 1). Nitrotyrosine measured by ELISA in the liver was decreased in the quercetin (340.6±47.6 ng/g protein; \( p < 0.01 \)) and clodronate (301.4±26.2 ng/g protein; \( p < 0.01 \)) groups compared with that in the LPS group (1,043±240 ng/g protein in the LPS group, Fig. 2-A). It was only 21.2±1.7 ng/g protein in the control group. The percentage of nitrotyrosine to total tyrosine measured by the HPLC-electrochemical detection method showed a similar trend (control: 0.0±0.01%, LPS: 2.0±0.27%, quercetin: 0.2±0.02%, clodronate: 0.1±0.02%, Fig. 2-B).

Histological examination revealed fatty degeneration of hepatocytes and enlarged sinusoids in the...
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The damage appeared to be more severe in the central venous area than in the portal venous area. In the quercetin and clodronate groups, the tissue damage was mild and limited to the central venous area. Particularly no damage was observed in the portal venous area of the clodronate group (Fig. 3). Plasma nitrite and nitrate levels were increased to 1,142±23 in the LPS group (29±4 in the control group) and mildly attenuated in the quercetin and clodronate group (724±16 and 619±6, respectively) (Fig. 4).

Plasma TNF-α level

The plasma TNF-α level was markedly elevated in the LPS group (1,124.1±167.6 pg/ml) and remained high (1,292.4±159.9 pg/ml) in the quercetin group, which contrasted the significantly low level (34.8±5.3 pg/ml) in the clodronate group (Fig. 5). The TNF-α level was very low in the control group (0.5±0.1 pg/ml).

Discussion

We found that ALT release and histological change induced by LPS were associated with nitrotyrosine formation. Treatment with a nitration inhibitor, quercetin or a Kupffer cell remover, clodronate prevented the damage and the nitrotyrosine formation.

As quercetin is a potent inhibitor of nitration, and was shown to be 10-fold more effective than another potent antioxidant ebselen [17, 28], its effect on the reduction of nitrotyrosine was expected. The association of the reduction of nitrotyrosine with the attenuation of the liver injury suggests pathognomonic role of nitration. Furthermore, almost complete protection against injury and reduction of nitrotyrosine by clodronate indicates that Kupffer cells may be the major cells responsible for the liver injury, as well as the nitrotyrosine formation. This is reasonable, since Kupffer cells can produce both NO and superoxide, which yields peroxynitrite [29, 30].

An important observation is that the level of TNF-α remained elevated in the case of quercetin treatment, even though the injury was markedly attenuated. This suggests that TNF-α itself is not pathogenic, but rather, an active species downstream of TNF-α is the culprit. This is in agreement with previous studies in patients and animal models, in which treatment with anti-TNF-α did not ameliorate the injury [5, 6, 31]. The marked reduction of TNF-α by clodronate indicates that Kupffer cells are a major source of TNF-α production. As clodronate has been used therapeutically to inhibit osteoclast-mediated bone resorption [32] and to reduce new metastases in breast cancer with a low rate of side effects [33], clodronate treatment may have potential for the amelioration of liver injury caused by septic shock. The reduction in plasma nitrite and nitrate concentrations by both treatments was less compared with the reduction in nitrotyrosine. This can be explained by the facts that quercetin is the inhibitor of nitrotyrosine formation and not NO inhibitor, and similarly clodronate depletes Kupffer cells but does not affect hepatocytes, sources of NO.

It is noteworthy that sinusoid expansion and fatty
degeneration of hepatocytes were improved by both treatments in the portal venous area, but these changes could not be completely eliminated in the central venous area. This regional difference in response can be explained by the fact that intraperitoneally injected quercetin is absorbed and reaches the liver via the portal venous system, resulting in a higher concentration in the portal region than in the central region. In the case of Kupffer cell removal by clodronate, a similar effect may occur, although more Kupffer cells reside in the portal area than in the central area.

Conclusion

This study demonstrated that LPS-induced liver injury involves nitrotyrosine formation and its inhibition protects the injury. The nitrotyrosine formation appears to be a downstream mechanism following TNF-α production. The observed effects of quercetin and clodronate may provide a rational basis for the development of novel and effective therapies to treat liver injury caused by septic shock.

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