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Ascorbic acid deficiency increases endotoxin influx to portal blood and liver inflammatory gene expressions in ODS rats

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ABSTRACT

Objective: The aim of this study was to determine whether ascorbic acid (AsA) deficiency-induced endotoxin influx into portal blood from the gastrointestinal tract contributes to the inflammatory changes in the liver.
Method: The mechanisms by which AsA deficiency provokes inflammatory changes in the liver were investigated in Osteogenic Disorder Shionogi (ODS) rats (which are unable to synthesize AsA). Male ODS rats (6-wk-old) were fed a diet containing sufficient (300 mg/kg) AsA (control group) or a diet without AsA (AsA-deficient group) for 14 or 18 d.
Results: On day 14, the hepatic mRNA levels of acute-phase proteins and inflammation-related genes were significantly higher in the AsA-deficient group than the control group, and these elevations by AsA deficiency were exacerbated on day 18. The serum concentrations of interleukin (IL)-1β and IL-6, which induce acute-phase proteins in the liver, were also significantly elevated on day 14 in the AsA-deficient group compared with the respective values in the control group. IL-1β mRNA levels in the liver, spleen, and lung were increased by AsA deficiency. Moreover, on both days 14 and 18, the portal blood endotoxin concentration was significantly higher in the AsA-deficient group than in the control group, and a significant correlation between serum IL-1β concentrations and portal endotoxin concentrations was found in AsA-deficient rats. In the histologic analysis of the ileum tissues, the number of goblet cells per villi was increased by AsA deficiency.
Conclusion: These results suggest that AsA deficiency-induced endotoxin influx into portal blood from the gastrointestinal tract contributes to the inflammatory changes in the liver.

Introduction

Ascorbic acid (AsA; i.e., vitamin C), is a water-soluble antioxidant and enzyme cofactor in humans and animals. Humans, other primates, guinea pigs, and Osteogenic Disorder Shionogi (ODS) rats [1,2] cannot synthesize AsA because they lack L-gulono-γ-lactone oxidase (EC.1.1.3.8), which catalyzes the terminal step of AsA biosynthesis. It is well known that AsA protects the body against oxidative stresses, such as inflammation. It has been demonstrated that AsA deficiency provoked hepatic inflammatory changes in ODS rats [3–6], a useful model of investigating the physiological role of AsA in humans, in light of the model's similar defect of the enzyme for AsA biosynthesis. As a consequence of AsA deficiency in ODS rats, their serum proinflammatory interleukin (IL)-6 [5] and cytokine-induced neutrophil chemoattractant-1 [6] concentrations are elevated, and the hepatic acute-phase protein (APP) expressions are stimulated [5]. However, it is unclear how AsA deficiency is linked to these inflammatory changes in the serum and liver.

The results of human and animal studies have shown that endotoxin can diffuse from the gut to the circulatory system in response to type of diet. For instance, excessive fat intake causes

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an increase in gut permeability, endotoxemia, and the stimulation of proinflammatory cytokine expressions, leading to low-grade inflammation in human and experimental animals [7–9]. Endotoxin is a component of the outer membrane of the cell wall of gram-negative bacteria, and lipopolysaccharide (LPS) is its active molecule. LPS in the blood stimulates the expression of proinflammatory cytokines including IL-1β and IL-6 in immune cells, and these cytokines induce inflammatory changes in the liver and other tissues [10]. The inflammatory changes in the serum and liver observed in AsA-deficient ODS rats were similar to those in LPS-induced low-grade inflammation. However, it has not been clarified whether AsA deficiency induces endotoxemia. In the present study, we hypothesized that AsA deficiency causes endotoxin invasion into portal blood, resulting in the induction of proinflammatory cytokine production and hepatic inflammatory responses.

To test our hypothesis, we examined the portal blood endotoxin concentration in AsA-deficient ODS rats, and we explored its relevance to AsA deficiency-stimulated proinflammatory cytokine production and hepatic inflammatory gene expression. We report here that AsA deficiency induces endotoxin influx into portal blood, and this endotoxemia might contribute to the inflammatory changes in the liver.

Materials and methods

Animals and diets

Male ODS (ODS/Shi Jcl-*od/od*) rats, 5 wk of age, were purchased from Japan CLEA (Tokyo). They were housed in individual, wire screen-bottomed cages in the animal colony of Nagoya University and maintained at 24°C with a 12-h light cycle (lights on from 08:00 to 20:00). The rats were allowed free access to water and a purified diet. The compositions of the basal and ascorbic-free diets are shown in Table 1. The dietary addition of 300 mg of AsA/kg diet is sufficient for maximum growth and prevents the development of scurvy in ODS rats [11].

All rats were fed the basal diet for 7 d before the start of the experiment, and then either the basal diet (control group) or the AsA-free diet (AsA-deficient group). In all experiments, rats were sacrificed by decapitation between 10:00 and 11:00. The animal care and experimental procedures were approved by the Animal Research Committee of Nagoya University (approval number 2013022801) and were conducted according to the Regulations for Animal Experiments at Nagoya University.

Experimental protocol

Experiment 1

During the experimental period, rats were fed a basal diet containing 300 mg AsA/kg diet (control group) or a diet without AsA (AsA-deficient group). It was observed previously that the growth rate of ODS rats fed the AsA-deficient diet begins to decrease on day 14 compared with controls [11]. In the present study, six rats from each group were sacrificed on the morning of days 14 and 18. The food intake of the AsA-deficient rats began to decrease slightly on day 12. Therefore, rats in the control group were pair-fed the mean amount consumed by rats in the AsA-deficient group from day 12. Blood was collected and serum was prepared by centrifugation at 1500*g* for 10 min and stored at −80°C. Tissues (liver, spleen, lung, epididymal fat tissue, ileum, kidney, adrenal gland, and aorta) were removed and frozen immediately in liquid nitrogen, and stored at −80°C.

Table 1
Compositions of basal diet and AsA-free diet

Component	Basal diet	AsA-free diet
	(G/kg diet)	
Casein	250	250
Mineral mixture*	35	35
Vitamin mixture†	10	10
Choline chloride	2	2
Corn oil	50	50
Cellulose powder	40	40
Sucrose	204.2	204.5
Cornstarch (α-starch)	408.5	408.5
Ascorbic acid	0.3	0

* AIN93-MX mineral mixture.
† AIN93-VX vitamin mixture.

Experiment 2

As the portal blood endotoxin concentrations of the greater part of control rats were lower than the minimum detection level of the present method, we performed Experiment 2 by using a sufficient number of rats (12–18 rats) in each group to detect the difference in the portal blood endotoxin concentration between the control and AsA-deficient groups. As in Experiment 1, rats in the control and AsA-deficient groups were fed their respective diets for 14 or 18 d. The number of each group was as follows: day 14 control group, n = 12; day 14 AsA-deficient group, n = 15; day 18 control group, n = 16; day 18 AsA-deficient group, n = 18. On day 14 or 18, rats were anesthetized and underwent a laparotomy through a large midline incision under aseptic conditions, and then 150 μL of the portal blood was collected by a needle with a heparinized syringe into pyrogen-free glass tubes and centrifuged at 200*g* for 20 min. The plasma was collected and stored at 4°C in pyrogen-free glass tubes until the endotoxin was measured. Finally, both plasma samples derived from Experiments 1 and 2 were used for the measurement of endotoxin concentration. On day 14, serum samples collected from 10 control group rats and 11 AsA-deficient rats were used to measure serum concentrations of IL-1β and IL-6.

Determination of tissue AsA concentration

The liver, spleen, and ileum were homogenized in ice-cold 50 g/L metaphosphoric acid and centrifuged for 10 min at 1600*g*. The AsA concentration in the supernatant was measured by the 2,4-dinitrophenylhydrazine method, with a modification in which the oxidation of AsA was accomplished with 2,6-dichlorophenolindophenol [12].

RNA preparation and gene expression analysis

Total RNA was extracted from frozen tissues using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). It was then treated with DNase using a TURBO DNA-free kit (Ambion, Carlsbad, CA, USA). Complementary Deoxyribonucleic Acide (cDNA) was synthesized using a high-capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA, USA). Gene expression was quantified by real-time polymerase chain reaction (PCR) using an ABI 7300 real-time PCR system with the Thunderbird qPCR Mix or the Thunderbird SYBR qPCR Mix (Toyobo, Osaka, Japan). TaqMan primers and probes were used to determine the level of IL-6 (TaqMan probe number, Rn.00561420_m1), tumor necrosis factor (*TNF*)-α (Rn.999999017_m1), C-reactive protein (*CRP*; Rn.00567307_g1) and *IL-1β* (Rn.01514151_m1; Predeveloped TaqMan Assay Reagents; Applied Biosystems). The primers used in the SYBR Green assay for *β-actin*, α1-acid glycoprotein (*AGP*), haptoglobin (*HP*), *IL-18*, monocyte chemoattractant protein 1 (*MCP-1*), inducible nitric oxide synthase (*iNOS*), and heme oxygenase 1 (*HO-1*) are shown in Table 2. The level of each mRNA was normalized to that of the corresponding β-actin.

Table 2
Primers for SYBR green assay

β-actin	
Sense	TTCAACACCCCAGCCATGT
Antisense	CAGAGGCATACAGGGACAACAC
AGP	
Sense	ACCCAGAACCTGCCAACATC
Antisense	GAGCCATTTCAGGGTCTCATTG
HP	
Sense	TGTGGGAAGCCCAAGCA
Antisense	TCCATGGAACCACCGATGAT
IL-18	
Sense	AAACCCGCCTGTGTTCGA
Antisense	TGGGATTCGTTGGCTGTTC
MCP-1	
Sense	ACGCTTCTGGGCCTGTTG
Antisense	GGCTGAGACAGCACGTGGAT
iNOS	
Sense	AGATCCGGTTCACAGTCTTGGT
Antisense	ACCTTCCGCATTAGCACAGAA
HO-1	
Sense	TCGGTAGAGGCGGCTGTTC
Antisense	GTCAACATGGACGCCGACTA

AGP, α1-acid glycoprotein; HO, heme oxygenase; HP, haptoglobin; IL, interleukin; iNOS, inducible nitric oxide synthase; MCP, monocyte chemoattractant protein