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Effects of globin digest and its active ingredient Trp-Thr-Gln-Arg on galactosamine/lipopolysaccharide-induced liver injury in ICR mice

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ABSTRACT

Aims: We investigated the effects of globin digest (GD) and its active ingredient Trp-Thr-Gln-Arg (WTQR) on galactosamine/lipopolysaccharide (GalN/LPS)-induced liver injury in imprinting control region (ICR) mice.
Main methods: The effects of WTQR and GD on the liver injury were examined by measuring the survival rate, serum aminotransferase activities, hepatic components, antioxidant enzyme activities, histopathological analysis, serum levels and hepatic gene expression of tumor necrosis factor-alpha (TNF-α), macrophage inflammatory protein-2 (MIP-2), and nitric oxide (NO) or inducible nitric oxide synthase (iNOS), and nuclear factor-kappa B (NF-κB) p65 content in GalN/LPS-treated ICR mice. RAW264 mouse macrophages were used to confirm the anti-inflammatory effects of WTQR and GD on the macrophages.
Key findings: WTQR and GD increased the survival rate, suppressed the serum aminotransferase activities, serum levels and hepatic gene expression of TNF-α, MIP-2, and NO or iNOS, and nuclear NF-κB p65 content in GalN/LPS-treated mice; decreased the oxidized glutathione content, increased the superoxide dismutase activity, and decreased the histopathological grade values of the hepatocyte necrosis and lobular inflammation in GalN/LPS-injured liver; and suppressed the release levels and gene expression of TNF-α, MIP-2, and NO or iNOS, and nuclear NF-κB p65 content in LPS-stimulated RAW264 macrophages. WTQR and GD may improve the antioxidant defense system and inflammatory status in GalN/LPS-injured liver.
Significance: These findings indicate that WTQR and GD have hepatoprotective effects on GalN/LPS-induced liver injury in ICR mice.

Introduction

Globin digest (GD), an oligopeptide mixture derived from edible globin proteins, improved hyperlipidemia and hyperglycemia in humans and other mammals, and has been used as a specified health food (Kagawa et al., 1996, 1998, 1999, 2008; Kamei et al., 2001; Inagaki et al., 2002; Okuda et al., 2004; Xu, 2008a, 2008b; Tong et al., 2008; Sasakawa et al., 2008; Nakaoka et al., 2010).

GD also improved the galactosamine (GalN)-induced liver injury in Sprague Dawley (SD) rats (Yamamoto et al., 2011). By screening imprinting control region (ICR) mice with GalN/lipopolysaccharide (LPS)-induced liver injury, Trp-Thr-Gln-Arg (WTQR) was found as a main active ingredient of GD (unpublished results). A low dose of LPS, an outer membrane component of gram-negative bacteria, in combination with GalN, a hepatotoxin, has been shown to induce an experimental liver injury that is similar to clinical acute hepatic failure (Galanos et al., 1979; Nakama et al., 2001). GalN mainly causes liver injury via the generation of free radicals and depletion of uridine triphosphate nucleotides (Sinha et al., 2007; Keppler et al., 1970), leading to inhibition of RNA and protein synthesis (Plaa, 1991; Koff et al., 1971; Shinozuka et al., 1973), and induction of apoptosis in the liver (Tsutsui et al., 1997; Muntané et al., 1998; Sun et al., 2003; El-Mofty et al., 1975). LPS directly activates macrophages, including Kupffer cells, the resident macrophages in the liver, to produce inflammatory cytokines, including tumor necrosis factor-alpha (TNF-α), macrophage inflammatory protein-2 (MIP-2), and nitric oxide (NO), causing hepatic injury (Bradham et al., 1998; Deutschman et al., 1996; Li et al., 2004; Morikawa et al., 1999). GD improved the inflammatory status and antioxidant status in GalN-injured liver of rats (Yamamoto et al., 2011). We assumed that WTQR and GD would also improve the inflammatory status and antioxidant status in GalN/LPS-injured liver of ICR mice, and examined as follows; we attempted to clarify the effects of WTQR and GD on the survival, serum aminotransferase activities, hepatic triglyceride (TG), total cholesterol (T-CHO), and thiobarbituric acid reactive substance (TBARS) (an indicator of lipid peroxidation) contents, inflammatory status by measuring serum levels and hepatic gene expression of TNF-α, MIP-2, and NO or inducible nitric oxide synthase (iNOS), and nuclear factor-kappa B (NF-κB) p65 content, and antioxidant status by measuring hepatic antioxidants such as reduced glutathione (GSH) and oxidized

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glutathione (GSSG), and antioxidant enzymes such as superoxide dismutase (SOD) (an enzyme to scavenge O_2^-), catalase (CAT) (an enzyme to decompose H_2O_2), glutathione peroxidase (Gpx) (an enzyme to metabolize both H_2O_2 and lipid hydroperoxides using GSH as a cosubstrate), and glutathione reductase (GR) (an enzyme to reduce GSSG to GSH) in GalN/LPS-treated mice. Caffeine had a hepatoprotective effect on GalN/LPS-treated rats (Akashi et al., 2009), and was used as the positive control. RAW264 mouse macrophages were used to confirm the anti-inflammatory effects of WTQR and GD on the macrophages. Dexamethasone (DX) suppressed the release levels of TNF-α and MIP-2 by LPS-stimulated RAW264.7 mouse macrophages (Sakai et al., 1997), and was used as the positive control.

Materials and methods

Materials

GD (MG Pharma, Osaka, Japan) and WTQR (c-Strong, Shanghai, China) were used for the examination. GD is produced from the enzymatic hydrolysis of globin protein in bovine or swine hemoglobin (Kagawa et al., 1991). The digest is an oligopeptide mixture consisting of 3–5 amino acid residues. GD is composed of more than 91% proteins and less than 8% free amino acids, and the molecular weights of the peptides contained range from 100 to 1500 u. WTQR is at positions 38–41 of the β-globin chain and is included in GD at 1.89%. GD, WTQR, and caffeine (WAK1707, Wako, Osaka, Japan) were dissolved in distilled water for in vivo examination. GD was administered orally to mice at 500, 1000, and 2000 mg/kg. WTQR was administered orally to mice at 25, 50, and 100 mg/kg. Caffeine was administered orally to mice at 100 mg/kg. The administering capacity of GD, WTQR, and caffeine was 10 ml/kg. D-galactosamine hydrochloride (GalN) (079-02054, Wako, Osaka, Japan) and LPS (127-05141, Wako, Osaka, Japan) were dissolved into saline and administered intraperitoneally to mice at 700 mg/kg and 10 μg/kg, respectively. The administering capacity of GalN/LPS was 5 ml/kg. For in vitro examination, GD was dissolved in Dulbecco's Modified Eagle's Medium (D-MEM) (08458-45, Nacalai Tesque, Kyoto, Japan) supplemented with 10% fetal bovine serum (FBS) (6 C0282, Nichirei, Tokyo, Japan), and used at doses of 0.5, 1, 2, 4, 8, and 12 mg/ml. WTQR was also dissolved in D-MEM supplemented with 10% FBS, and used at doses of 0.05, 0.1, 0.25, 0.5, 1, and 2 mg/ml. DX (11107-51, Nacalai Tesque, Kyoto, Japan) was dissolved in ethanol to 10 mM, diluted with D-MEM supplemented with 10% FBS, and used at a dose of 10 μM.

Animals and treatments

The present study conformed to the ethical guidelines for animal experimentation of MG Pharma Inc., which is in accordance with the Declaration of Helsinki. ICR strain male mice (age, 7 weeks; weight, 31.3–38.4 g) (Japan SLC, Shizuoka, Japan) were used. The mice were housed in an air-conditioned room (23 ± 2 °C, 50 ± 10% RH) with a 12-h light and dark cycle (7:00–19:00 light hours). The mice were kept in an experimental animal room for 7 days with free access to Certified Diet-MF (Oriental Yeast, Tokyo, Japan) and water. Mice with pelage in good condition were used for the experiments.

GalN/LPS-induced liver injury in ICR mice

ICR mice were divided into the following nine groups at random, with the number of mice per group shown in the respective table for each experiment (Tables 1–7): vehicle control; GalN/LPS control; 25, 50, and 100 mg/kg WTQR; 500, 1000, and 2000 mg/kg GD; and 100 mg/kg caffeine. GalN/LPS was administered intraperitoneally to mice of the GalN/LPS control, WTQR, GD, and caffeine groups. Saline was administered intraperitoneally to mice of the vehicle control group. Distilled water was administered orally to mice of the vehicle control group at 1 h before saline administration. Distilled water was administered orally to mice of the GalN/LPS control group at 1 h before GalN/LPS administration. WTQR, GD, or caffeine was administered orally to mice at 1 h before GalN/LPS administration. The administration time of the test articles followed the previously described method (Kim et al., 2008, 2010). The mice were fasted after administration of the test articles. At 1.5 h or 16 h after GalN/LPS administration, blood was drawn from the inferior vena cava under nembutal anesthesia. Mice were then euthanized by blood removal from the inferior vena cava, and the liver was washed in saline, wiped, and weighed. For hepatic components and antioxidant enzymes, the left hepatic lobe was divided in accordance with the respective instruction manual at 16 h after GalN/LPS administration, and frozen with liquid N_2, and kept at −80 °C until used. For histopathological analysis, half of the right hepatic lobe was fixed in 10% phosphate-buffered formalin until used at 16 h after GalN/LPS administration. For quantitative real-time reverse transcriptase polymerase chain reaction (RT-PCR), about 30 mg of the right hepatic lobe was cut into about 3 mm cubes at 1.5 h or 16 h after GalN/LPS administration, and soaked in RNAlater Solution (AM7024, Ambion, TX, USA) overnight at 4 °C, and then stored frozen at −30 °C until used. For nuclear NF-κB p65 content, about 300 mg of the right hepatic lobe was used immediately in accordance with the instruction manual at 1.5 h after GalN/LPS administration.

Table 1
Effects of WTQR and GD on the survival in GalN/LPS-treated mice.

Group	Number of mice (surviving/total)				Survival rate (%)
	Experiment number				
	1	2	3	Total	Total
Vehicle control	4/4	5/5	4/4	13/13	100
GalN/LPS control	5/10	6/10	7/10	18/30	60
GalN/LPS + 25 mg/kg WTQR	–	–	9/10	9/10	90
GalN/LPS + 50 mg/kg WTQR	8/10	7/10	–	15/20	75
GalN/LPS + 100 mg/kg WTQR	8/10	–	7/10	15/20	75
GalN/LPS + 500 mg/kg GD	8/10	–	–	8/10	80
GalN/LPS + 1000 mg/kg GD	8/10	–	–	8/10	80
GalN/LPS + 2000 mg/kg GD	9/10	7/10	–	16/20	80
GalN/LPS + 100 mg/kg caffeine	8/10	–	–	8/10	80

The survival at 16 h after GalN/LPS administration in all three experiments is shown.

LPS-stimulated RAW264 mouse macrophages

RAW264 mouse macrophages (RCB0535, RIKEN, Ibaraki, Japan) were seeded at 5×10^4 cells/well in a 24-well plate for release levels of TNF-α, MIP-2, and NO, or at 2.5×10^5 cells/well in a 6-well plate for quantitative real-time RT-PCR and nuclear NF-κB p65 content, and incubated overnight at 37 °C under 5% CO_2 in D-MEM supplemented with 10% FBS. The following day, cells were cultured with 0.05, 0.1, 0.25, 0.5, 1, or 2 mg/ml WTQR, 0.5, 1, 2, 4, 8, or 12 mg/ml GD, or 10 μM DX for 24 h, and then with 100 ng/ml LPS and above-mentioned test articles for 1.5 h or 24 h at 37 °C under 5% CO_2 in D-MEM supplemented with 10% FBS. For release levels of TNF-α, MIP-2, and NO, cell supernatant was collected at 24 h after LPS stimulation, and then stored frozen at −30 °C until used. For quantitative real-time RT-PCR and nuclear NF-κB p65 content, cells were used immediately in accordance with the respective instruction manual at 1.5 h or 24 h after LPS stimulation.

Serum aminotransferase activities

The serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) activities were measured using a Transaminase CII-Test Wako (431-30901, Wako, Osaka, Japan). In all the measurement items, each sample was diluted and measured within the detection range in accordance with the respective instruction manual.