

***Pyropia yezoensis* glycoprotein regulates antioxidant status and prevents hepatotoxicity in a rat model of D-galactosamine/lipopolysaccharide-induced acute liver failure**

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Received January 24, 2015; Accepted November 18, 2015

DOI: 10.3892/mmr.2016.4932

Abstract. The present study aimed to investigate the effects of *Pyropia yezoensis* glycoprotein (PYGP) on hepatic antioxidative enzyme activity and mitogen-activated protein kinase (MAPK) phosphorylation in a rat model of D-galactosamine/lipopolysaccharide (D-GalN/LPS)-induced hepatotoxicity. Glutamic-oxaloacetic transaminase (GOT) and glutamic-pyruvic transaminase (GPT) were measured to determine the severity of hepatotoxicity. Treatment with D-GalN/LPS significantly increased the GOT, GPT and lipid peroxidation levels, and decreased the antioxidant capacity of the rats. Treatment with PYGP (150 and 300 mg/kg/body weight) decreased the levels of GOT, GPT and lipid peroxidation levels. The activities of antioxidative enzymes, including catalase, glutathione S-transferase and glutathione were upregulated following PYGP treatment. Furthermore, D-GalN/LPS-induced MAPK phosphorylation, and inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) protein expression were downregulated by PYGP. These results indicated that PYGP may exert hepatoprotective effects via the upregulation of antioxidative enzymes, and the downregulation of the MAPK signaling pathway and iNOS and COX-2 expression.

Introduction

Acute liver failure (ALF), which is characterized by coagulopathy and encephalopathy, is associated with a high mortality rate (1,2). ALF may be induced by alcohol, viral hepatitis, bacteria or hepatotoxic drugs (3), and there is currently no available therapy for ALF other than liver transplantation (4).

D-galactosamine (D-GalN) and lipopolysaccharide (LPS) are often used to generate hepatitis test models (5). Animal models are used in hepatoprotective drug screening, and to

elucidate the mechanisms underlying clinical liver dysfunction (6). D-GalN induces a loss of uridine triphosphate via the galactose pathway, and inhibits RNA and protein synthesis (7), thus resulting in hepatic necrosis and apoptosis due to metabolic changes (8). LPS activates liver macrophages, which secrete diverse proinflammatory cytokines, thus inducing hepatic necrosis and reducing the production of antioxidant enzymes (9).

The D-GalN/LPS hepatotoxic model induces inflammatory reactions and oxidative stress within the liver (10,11), due to increased inflammation and expression of inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) (12). iNOS has an important role in drug-induced liver injury (13), and COX-2 has an essential role in D-GalN/LPS-induced inflammation (14). Inflammation leads to the production of reactive oxygen species (ROS), including H_2O_2 , O_2^- and OH^- (15). ROS attack polyunsaturated fatty acids in the cell membrane via lipid peroxidation, and trigger various pathological states, including oxidative stress (16).

Mitogen-activated protein kinases (MAPKs) comprise three major proteins: C-jun NH₂-terminal kinase (JNK), p38 MAPK and extracellular signal-regulated kinase (ERK). Phosphorylated MAPK proteins have various roles in oxidative stress and inflammatory diseases (17). In particular, activated JNK has an important role in hepatic injury via activation of the caspase cascade and induction of liver cell necrosis (18).

Pyropia yezoensis is a type of red algae, which has long been considered an important food source in Korea, Japan and China (19). Various previous studies have demonstrated the therapeutic effects of *P. yezoensis*, including chemoprotective (20), anticancer (21) and anti-inflammatory activities (22). There are currently no studies regarding the antioxidative activities of *P. yezoensis* glycoprotein (PYGP) against D-GalN/LPS-induced hepatotoxicity. The present study aimed to investigate the anti-inflammatory effects of PYGP against D-GalN/LPS *in vivo*.

Materials and methods

Preparation of PYGP. *P. yezoensis* was purchased in 2014 (Suhyup, Seoul, South Korea). *P. yezoensis* powder (40 g) was diluted with 1 L distilled water and stirred for 4 h at room temperature. The solution was then centrifuged at 3,000 x g and 4°C for 20 min, and vacuum filtered. Triple the volume of

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Key words: D-GalN/LPS, MAPK, glycoprotein, *Pyropia yezoensis* glycoprotein

ethanol was added to the solution (total quantity of filtrate x 3). After 24 h, the solution was filtered and concentrated using rotary evaporation at 40°C. The concentrated solution was divided into 1.5 ml tubes, freeze-dried, and stored at -70°C until further use.

Experimental animals. Male Sprague-Dawley rats (6 weeks old) were purchased from Samtaco (Osan, South Korea). Animal studies were conducted in accordance with the Animal Ethics Committee of the Pukyong National University (Busan, South Korea). The rats were maintained in the following laboratory conditions: 23±3°C, 12 h light/12 h dark cycle and 50% humidity, with *ad libitum* access to food and water.

Experimental design. The rats were randomly divided into four groups (n=5/group): Group 1, control rats received distilled water only; group 2, rats received 500 mg/kg/body weight (BW) D-GalN + 10 µg/kg/BW LPS; group 3, rats received 500 mg/kg/BW D-GalN + 10 µg/kg/BW LPS + 150 mg/kg/BW PYGP; and group 4, rats received 500 mg/kg/BW D-GalN + 10 µg/kg/BW LPS + 300 mg/kg/BW PYGP. PYGP was administered orally once a day for 7 days. Hepatotoxicity was induced in the rats by intraperitoneal injection of D-GalN/LPS (Sigma-Aldrich, St. Louis, MO, USA) at a dose of 500 mg/kg/BW D-GalN and 10 µg/kg/BW LPS. The rats were sacrificed under mild ether anesthesia (Duksan Pure Chemicals Co., Ltd., Ansan, South Korea) by decapitation for blood and liver sample collection 6 h after induction of hepatotoxicity.

GOT/GPT measurement. The blood samples were centrifuged at 3,000 x g for 20 min at 4°C to collect serum and stored at -20°C until analysis. The activities of glutamic-oxaloacetic transaminase (GOT) and glutamic-pyruvic transaminase (GPT) in the serum samples were determined using an enzymatic analysis kit (Asan Pharmaceuticals, Hwasung, South Korea), according to the manufacturer's protocols. The absorbance was measured at 505 nm using a microplate reader (Benchmark Plus 10730; Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Lipid peroxidation measurements. The liver tissues were added to 1X butyl hydroxyl toluene (Cell Biolabs, Inc., San Diego, CA, USA) and homogenized on ice at 10,000 x g for 5 min to collect the supernatant. According to the Thiobarbituric Acid Reactive Substances (TBARS) Assay kit protocol (Cell Biolabs), 100 µl of sample or malondialdehyde (MDA) standard was added to microcentrifuge tubes and then 100 µl SDS lysis solution was added, mixed thoroughly, incubated for 5 min at room temperature, and 250 µl of TBA reagent added. Each tube was closed, incubated at 95°C for 60 min, removed and then cooled to room temperature in an ice bath for 5 min. All the sample tubes were centrifuged at 842 x g for 15 min, the supernatant removed, 200 µl was transferred, along with 200 µl of MDA standard, to a 96-well microplate compatible with a microplate reader (Benchmark Plus 10730) and the absorbance read at 532 nm.

Antioxidant enzyme measurements. Antioxidant enzyme activities, including catalase (CAT), glutathione (GSH) and

glutathione S-transferase (GST), were measured in the liver samples using appropriate kits, according to the manufacturer's protocols (Catalase Assay kit, Glutathione Assay kit and Glutathione S-Transferase Assay kit; all Cayman Chemical Company, Ann Arbor, MI, USA). The absorbance was measured using a microplate reader (Benchmark Plus 10730).

Western blot analysis. Liver tissue samples were homogenized in lysis buffer [150 mM sodium chloride, 50 mM Tris-HCl (pH 7.5), 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 1% Triton X-100 and 2 mM ethylenediaminetetra-acetic acid; Intron Biotechnology, Inc., Seongnam, South Korea] containing inhibitors (1 mM Na₃VO₄, 1 µg/ml aprotinin, 1 µg/ml leupeptin, 1 µg/ml pepstatin A and 1 mM phenylmethylsulfonyl fluoride; Sigma-Aldrich). Protein concentration was determined using the Bichinchoninic Acid Assay kit (Pierce Biotechnology, Inc., Rockford, IL, USA). Equal protein quantities (20 µg) from each sample were separated by 10-15% SDS-polyacrylamide gel electrophoresis and transferred to a polyvinylidene fluoride membrane (EMD Millipore, Billerica, MA, USA). The membrane was blocked with 1% bovine serum albumin (BSA) in TBST [10 mM Tris-HCl (pH 7.5), 150 mM NaCl and 0.1% Tween 20; USB Corporation, Cleveland, OH, USA]. Subsequently, the membrane was incubated for 4 h at room temperature with the following primary immunoglobulin G antibodies, diluted to 1:1,000 in BSA/TBST: Rabbit anti-rat ERK polyclonal antibody (cat. no. sc-94), rabbit anti-rat phosphorylated (p)-ERK polyclonal antibody (cat. no. sc-7383), mouse anti-rat JNK monoclonal antibody (cat. no. sc-7345), mouse anti-rat p-JNK monoclonal antibody (cat. no. sc-6254), rabbit anti-rat p38 polyclonal antibody (cat. no. sc-7149), mouse anti-rat p-p38 monoclonal antibody (cat. no. sc-7973), mouse anti-rat iNOS polyclonal antibody (cat. no. sc-650), goat anti-rat COX-2 polyclonal antibody (cat. no. sc-1745) and rabbit anti-rat GAPDH polyclonal antibody which served as a loading control (cat. no. sc-25778; all Santa Cruz Biotechnology Inc., Dallas, TX, USA). The membrane was then incubated with peroxidase-conjugated anti-goat (cat. no. 81-1620), anti-mouse (cat. no. 62-6520) and anti-rabbit (cat. no. 65-6120) secondary antibodies (1:10,000; Bethyl Laboratories, Inc., Montgomery, TX, USA) for 1 h at room temperature. Antibody binding was visualized using the Super Signal West Pico Stable Peroxide solution and the Super Signal West Pico Luminol/Enhancer solution (Thermo Fisher Scientific, Inc., Rockford, IL, USA). The signal was developed on Kodak X-ray film (Kodak, Rochester, NY, USA) using a developer and fixer twin pack (Kodak).

Statistical analysis. The results of the present study are presented as the mean ± standard deviation. Data were analyzed using SPSS version 10.0 software (SPSS, Inc., Chicago, IL, USA). Results were validated using analysis of variance and Duncan's multiple range test. P<0.05 was considered to indicate a statistically significant difference.

Results

PYGP suppresses GOT and GPT levels in the serum of D-GalN/LPS-treated rats. GOT and GPT serum levels are

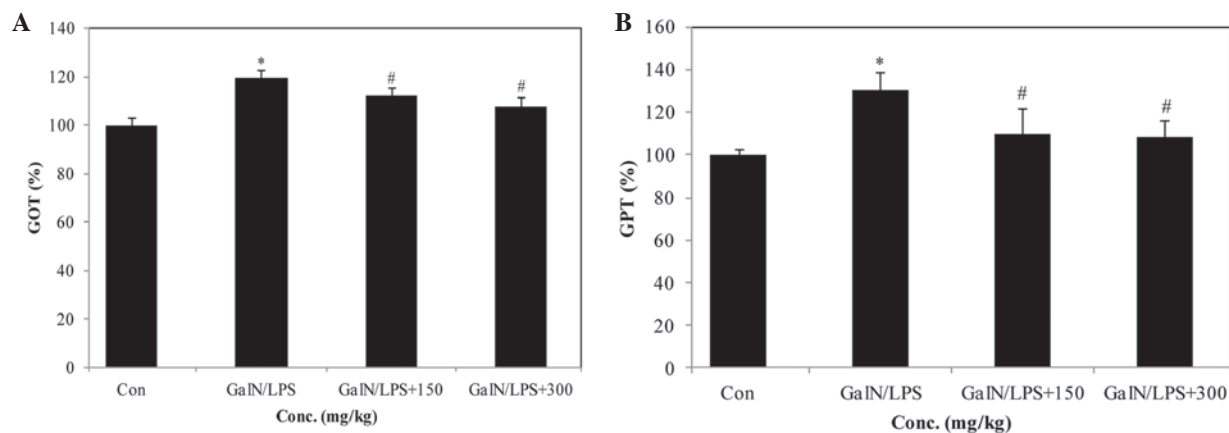


Figure 1. (A) GOT and (B) GPT levels in the serum of control and experimental rat groups. Data are presented as the mean \pm standard deviation. *P<0.05 vs. the control group, #P<0.05 vs. the GaIN/LPS group. GaIN, D-galactosamine; LPS, lipopolysaccharide; 150, 150 mg/kg/body weight PYGP; 300, 300 mg/kg/body weight PYGP; PYGP, *Pyropia yezoensis* glycoprotein; GOT, glutamic-oxaloacetic transaminase; GPT, glutamic-pyruvic transaminase; Con, control.

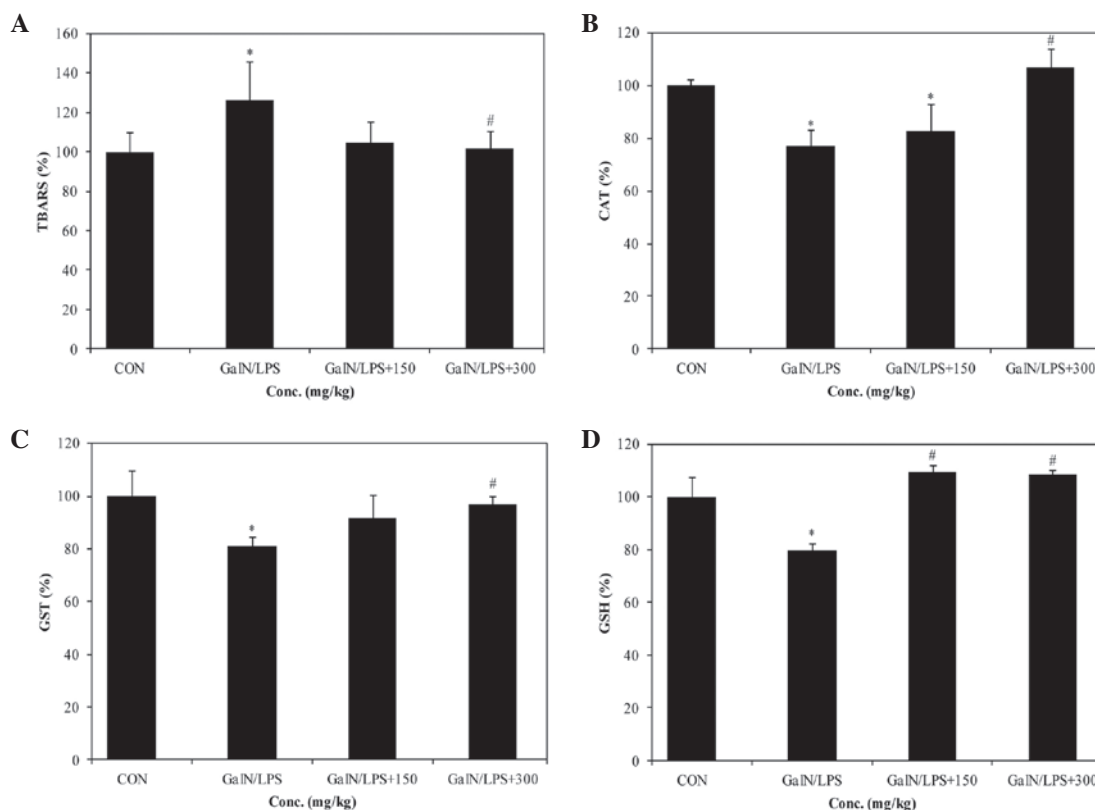


Figure 2. Levels of TBARS and antioxidant enzyme activity (CAT, GST and GSH) in the livers of control and experimental rat groups. (A) TBARS, (B) CAT, (C) GST and (D) GSH. Data are presented as the mean \pm standard deviation. *P<0.05 vs. the control group, #P<0.05 vs. the GaIN/LPS group. GaIN, D-galactosamine; LPS, lipopolysaccharide; 150, 150 mg/kg/body weight PYGP; 300, 300 mg/kg/body weight PYGP; PYGP, *Pyropia yezoensis* glycoprotein; TBARS, thiobarbituric acid reactive substances; CAT, catalase; GST, glutathione S-transferase; GSH, glutathione; Con, control.

important indicators of liver function (23). Injection with D-GaIN/LPS elevated the levels of GOT and GPT; however, treatment with 300 mg/kg/BW PYGP significantly reduced these levels (Fig. 1A and B).

Effects of PYGP on D-GaIN/LPS-induced oxidative stress and antioxidant enzyme activity. A total of 6 h post-D-GaIN/LPS injection, the TBARS levels were determined, which indicate liver tissue lipid peroxidation. As shown in Fig. 2A, TBARS increased significantly following

treatment with D-GaIN/LPS. Conversely, TBARS levels in the D-GaIN/LPS+PYGP 150 and D-GaIN/LPS+PYGP 300 groups were markedly decreased. Furthermore, anti-oxidant enzyme activities were markedly decreased in the D-GaIN/LPS group, as compared with in the control group. CAT levels were decreased by ~20%, as compared with the control group. In addition, GST and GSH levels were decreased following D-GaIN/LPS treatment, however, levels were restored to control group levels following treatment with PYGP (Fig. 2B-D).

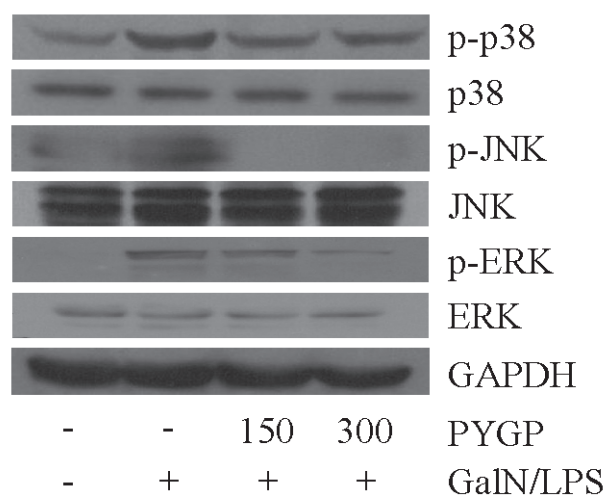


Figure 3. Expression levels of MAPK protein in the livers of control and experimental rat groups. Liver samples were homogenized in lysis buffer and western blotting was performed to determine the total protein expression and phosphorylation levels of ERK, JNK and p38 MAPK. PYGP, *Pyropia yezoensis* glycoprotein; GalN, D-galactosamine; LPS, lipopolysaccharide; MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; JNK, c-jun N-terminal kinase; p-, phosphorylated; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

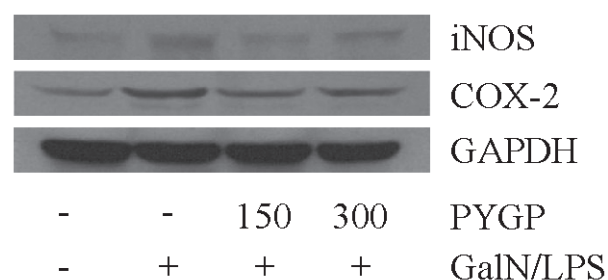


Figure 4. Expression levels of iNOS and COX-2 protein in the livers of control and experimental rat groups. Liver samples were homogenized in lysis buffer and western blotting was performed to determine the total protein expression levels of iNOS and COX-2. PYGP, *Pyropia yezoensis* glycoprotein; GalN, D-galactosamine; LPS, lipopolysaccharide; iNOS, inducible nitric oxide synthase; COX-2, cyclooxygenase-2 GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

PYGP suppresses D-GalN/LPS-induced MAPK phosphorylation. To investigate whether PYGP was able to modulate MAPK signaling, MAPK protein expression and phosphorylation levels were detected by western blot analysis. The protein expression levels of ERK, JNK and p38 did not differ between the groups. However, the phosphorylation of these proteins increased in the D-GalN/LPS-treated group, as compared with in the control group. In the D-GalN/LPS + PYGP co-treated groups, the phosphorylation levels of these proteins were downregulated (Fig. 3). These results suggest that PYGP may inhibit D-GalN/LPS-induced MAPK phosphorylation.

Effects of PYGP on iNOS and COX-2 protein expression. To confirm the effects of PYGP on inflammation in the rat liver, D-GalN/LPS-induced iNOS and COX-2 protein expression levels were detected. Following treatment with D-GalN/LPS, the protein expression levels were markedly increased; however,

treatment with PYGP prior to injection with D-GalN/LPS inhibited D-GalN/LPS-induced iNOS and COX-2 protein expression (Fig. 4). These results indicate that PYGP has an important role in the suppression of D-GalN/LPS-induced iNOS and COX-2 protein expression.

Discussion

Treating rats with a combination of D-GalN and LPS is widely used in studies researching the mechanisms underlying human ALF (24). D-GalN and LPS co-treatment induces greater critical hepatic damage, accompanied by apoptotic and necrotic changes in the liver, which closely resembles human viral hepatitis (25,26). In the present study, administration of D-GalN/LPS increased GOT and GPT serum levels; however, oral administration of PYGP attenuated these levels. These results suggested that D-GalN/LPS was able to induce severe damage to hepatic membranous tissues, and PYGP may prevent this hepatotoxicity.

D-GalN/LPS hepatotoxicity induces ROS production and reduces antioxidant enzyme activity in the liver (27). Furthermore, ROS may cause cell membrane lipid peroxidation (28). Oxidative stress is a well-known factor in D-GalN/LPS-induced liver injury. Increased TBARS and conjugated dienes have previously been detected following treatment with D-GalN/LPS (29). In the present study, the levels of TBARS increased in response to D-GalN/LPS treatment; however, co-treatment with D-GalN/LPS and PYGP suppressed hepatic TBARS levels. Antioxidant enzymes, including CAT, GST and GSH are important in D-GalN/LPS hepatotoxicity. CAT catalyzes the dismutation reaction of H_2O_2 , resulting in the formation of H_2O and O_2 (26). GSH is a substrate of GST, and GST catalyzes the conjugation of GSH with drugs and chemicals (30). In the present study, treatment with D-GalN/LPS significantly reduced the activities of CAT, GST and GSH, as compared with in the control group. Conversely, increased CAT and GST activities, and GSH levels were detected following PYGP treatment. These results suggested that PYGP exerts antioxidative effects against D-GalN/LPS-induced liver injury.

MAPKs comprise ERK, JNK and p38 proteins, which are phosphorylated by D-GalN/LPS (6). These proteins are involved in cell proliferation, differentiation, metabolism, survival and apoptosis (31). In particular, these proteins regulate cytokine production, and the expression of tumor necrosis factor- α and transcription factors (32,33). In the present study, treatment with PYGP significantly suppressed the GalN/LPS-induced phosphorylation of ERK, JNK and p38. These results indicated that GalN/LPS and PYGP co-treatment may reduce MAPK phosphorylation.

Inflammation occurs via various biological pathways. Nitric oxide (NO) production occurs via the iNOS pathway (34); in the cell, increased iNOS protein expression produces large amounts of NO, which increases the prevalence of inflammation (35). In addition, overexpression of NO induces hepatic dysfunction and hepatotoxicity (36). COX-2 is associated with the pathophysiology of inflammatory dysfunction, and the production of prostaglandins and thromboxanes (37), which may lead to hepatic injury (12). In the present study, PYGP pretreatment inhibited GalN/LPS-induced iNOS and COX-2 overexpression.

In conclusion, the present study demonstrated that PYGP may exert protective effects against D-GalN/LPS-induced ALF via inhibition of MAPK phosphorylation and iNOS and COX-2 expression. In addition, PYGP increased the activity of antioxidant enzymes.

Acknowledgements

The present study was supported by the Fishery Commercialization Technology Development Program through the Korean Institute of Planning and Evaluation of Technology in Food, Agriculture, Forestry and Fisheries (iPET) funded by the Ministry of Oceans and Fisheries (grant no. 2012300734).

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