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Protective Effects of Arabinogalactan-Peptide Isolated from Wheat Flour against Myocardial Injury in an Ischemia/Reperfusion Rat Model

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ABSTRACT: We have previously shown that supplementation of wheat with hot-water extract reduces myocardial injury by inhibiting apoptosis in a rat model of myocardial infarction (MI). Arabinogalactan-peptide (AGP), a cell wall polysaccharide of wheat, was also responsible for the protection. However, the underlying mechanisms were not elucidated. In this study, we investigated the underlying mechanisms for how AGP supplementation reduces myocardial injury. First, we isolated highly pure AGP from all-purpose wheat flour. We supplemented rats with AGP at a dose of 100 mg/kg/d for 3 days, and subjected the rats to ischemia (30 min) through ligation of the left anterior descending coronary artery followed by reperfusion (3 h) through a release of the ligation. Supplementation with AGP significantly reduced the infarct size in the heart. In addition, AGP intake inhibited the apoptotic cascade, determined through decreased mitogen-activated protein kinases (p38 and c-Jun N-terminal kinase) phosphorylation, decreased Bcl-2-associated X protein/B-cell lymphoma ratios, and decreased generation of nicked DNA, which was confirmed through western blotting and terminal deoxynucleotidyltransferase-mediated dUTP nick-end labeling staining. These findings indicate that AGP intake can protect against myocardial injury. Traditionally, consumption of dietary fiber such as AGP has been shown to reduce MI risk by inhibiting preocclusion steps through reducing risk factors. Our findings suggest that AGP intake can also reduce MI risk by inhibiting postocclusion steps. This study describes a better dietary recommendation and new prevention strategy for reducing MI risk through regular consumption of wheat rich in AGP.

Keywords: arabinogalactan protein, cardioprotectant, dietary fiber, ischemia/reperfusion, myocardial infarction

INTRODUCTION

Coronary heart disease (CHD), a major cause of death and disability worldwide. CHD manifests as angina pectoris and myocardial infarction (MI), of which MI is caused by extensive myocardial cell death in the heart (1). CHD is usually caused by atherosclerosis, which is initiated when low-density lipoproteins (LDL) accumulate and are oxidized in the lining of the coronary arteries, resulting from dysfunction of endothelial cells due to risk factors such as elevated LDL levels, diabetes, and elevated blood pressure (2,3). Accumulation of oxidized LDL eventually leads to atherosclerotic plaque formation. Plaque buildup in turn narrows the coronary arteries and leads to insufficient blood supply, and the resulting symptoms are referred to as angina pectoris (preocclusion steps) (4-6). Occasionally, the plaque ruptures and a thrombus subsequently forms in the lesion, resulting in artery occlusion. This occlusion event is responsible for ischemia downstream of the occlusion site (postocclusion steps). This state of hypoxia and hypoglycemia interrupts ATP production, resulting in activation of mitogen-activated protein kinases (MAPKs), including p38 and c-Jun N-terminal kinase (JNK), through phosphorylation. Activated MAPK triggers apoptotic cascades mediated by B-cell lymphoma (Bcl-2) and Bcl-2-associated X protein (Bax) (7). Eventually, myocardial cells in the lesion die through apoptosis and necrosis, and the extensive cell death leads to MI and, occasionally, sudden death. As many patients with MI die before receiving reperfusion therapy (8), development of improved prevention strategies will contribute to reducing deaths attributed to CHD.

Recent studies have demonstrated that plant-based diets that consist of certain foods, such as whole grains,

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fruits, vegetables, pulses, nuts, and seafood, are associated with a reduced risk of CHD mortality (9,10). Of the foods that constitute the plant-based diets, dietary fiber, of which cell wall polysaccharides account for the majority, is one active component responsible for the reduced risk of CHD mortality (11,12). Traditionally, reduction of CHD risk through consumption of plant-based diets (10) and dietary fiber (12) was thought to be associated with reduction of risk factors, such as dyslipidemia, hypertension, and diabetes, that affect the preocclusion steps through narrowing the coronary arteries. Recently, we showed that supplementation of foods components constituting plant-based diets (including grains such as wheat and barley, fruits such as apple and pear, vegetables such as red cabbage, pulses such as kidney bean, nuts such as almond, and seaweed such as kelp) protects against myocardial injury in a rat model of myocardial infarction (13). Of these food constituents, various cell wall polysaccharides showed efficacy in reducing myocardial injury. Supplementation of hot-water extract of wheat reduced myocardial injury by inhibiting apoptosis and subsequently delayed progression to heart failure. These findings suggest that consumption of plant-based diets and dietary fiber might reduce CHD risk by inhibiting postocclusion steps. Of the cell wall polysaccharides in wheat, those consisting of arabinose, such as arabinoxylan and arabinogalactan-peptide (AGP), were responsible for the protection. However, underlying mechanisms were not elucidated (13). In this study, we investigated the mechanisms underlying how AGP supplementation reduces myocardial injury through inhibiting apoptosis. In addition, we present procedures for isolation of AGP from wheat flour because AGP was not commercially available.

MATERIALS AND METHODS

Isolation and characterization of AGP

AGP was isolated from wheat flour as previously described but with a slight modification (14) (Fig. 1). Locally purchased all-purpose flour was extracted with 8-fold 80% (w/v) ethanol for 30 min at boiling temperature, and the collected flour was rinsed with 1.5-fold 80% ethanol. The flour was extracted with 6-fold ethanol for another 30 min at boiling temperature, from which deglutenized flour was obtained after centrifugation (14,000 *g*, 20 min, and 20°C), and was air-dried overnight (step 1). Finely ground deglutenized flour (200 g) was stirred with 1.6 L of pre-chilled water for 1 h at 4°C and centrifuged. The volume of the supernatant was reduced to 500 mL on a rotary evaporator (step 2). An appropriate amount of 95 % ethanol was added to the supernatant to make a solution containing 60% (w/v) ethanol. The solution was centrifuged, and the supernatant was reduced to 130 mL on a rotary evaporator (step 3). An appropriate amount of 95% ethanol was added slowly to the supernatant until the solution reached 80% (w/v) ethanol. After centrifugation, the precipitate containing AGP was recovered, washed two times with 95% ethanol, washed once with acetone, and air-dried to yield crude AGP (step 4). The crude AGP (400 mg) was dissolved in 15 mL of 0.1 M phosphate buffer (pH 7.15). The solution was slowly saturated with ammonium sulfate and stirred overnight (step 5). The solution was then filtered and dialyzed for 4 days. The

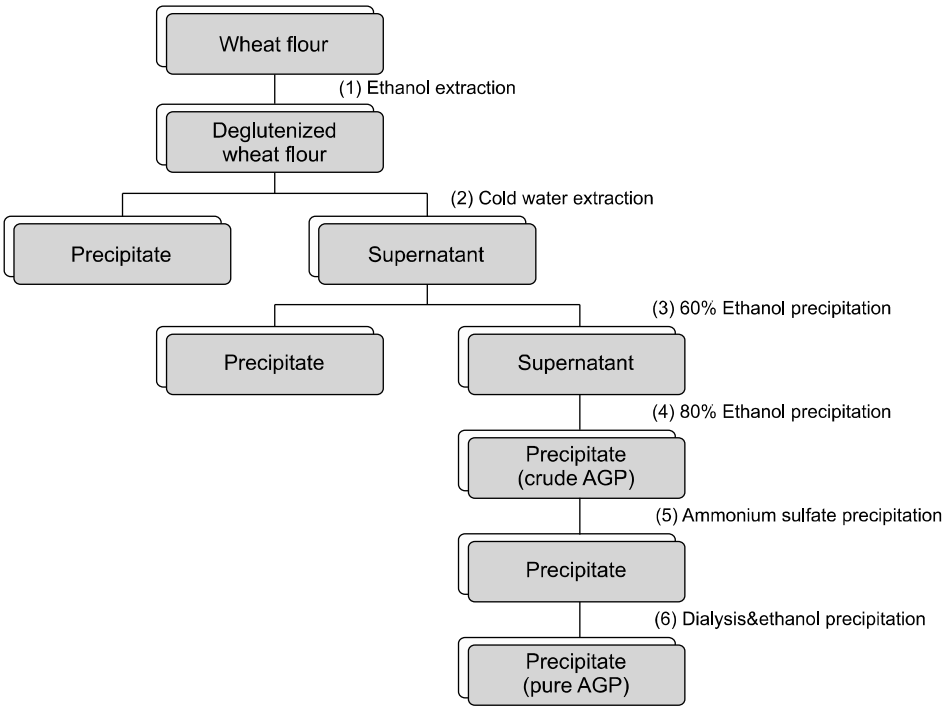

Fig. 1. Schematic presentation for the isolation of arabinogalactan-peptide (AGP) from wheat flour.

dialyzed solution was reduced to about 5 mL, and 95% ethanol was added dropwise to precipitate AGP. The precipitate was collected, washed with 95% ethanol, washed with acetone, and air-dried to yield AGP (step 6). AGP purity was confirmed through gel permeation chromatography (GPC) at Korea Polymer Technology Testing & Research Institute (Gwacheon, Korea), with the following running conditions: pure AGP was dissolved in a phosphate buffer (0.1 M $NaNO_3$+0.02 M Na_2HPO_4, pH 7.0) to 3 mg/mL and analyzed with a Waters GPC system (Milford, MA, USA) equipped with 2× TSKgel GMPWXL column (Tosoh Bioscience LLC., Tokyo, Japan). Samples (100 μL) were injected into the column and eluted with the buffer at a 1.0 mL/min flow rate at 45°C. The AGP peak was detected with a refractive index (RI) detector. Polysaccharides with known molecular weights were used as standards. The compositions of neutral monosaccharide in AGP were assessed as previously described (4). Briefly, AGP was hydrolyzed with trifluoroacetic acid, reduced with sodium borohydride, and acetylated with acetic anhydride to produce alditol acetate forms of the corresponding monosaccharides. Finally, the compositions of the alditol acetates in AGP were assessed through gas chromatography (Agilent 7890A, Agilent Technologies, Loveland, CO, USA).

Animals

Eight-week-old male Sprague-Dawley (SD) rats were purchased from Samtaco Inc. (Osan, Korea). Animal experiments were carried out according to the guidelines for animal care and use of laboratory animal protocols approved by the Institutional Animal Care and Research Advisory Committee of Catholic University, Daegu, Korea (DCIAFCR-151230-20-Y). The animals were housed with food and water available *ad libitum* under diurnal lighting conditions in a temperature-controlled environment until the experiment began.

Preparation and supplementation of diets

Diets containing AGP were prepared and supplemented as described previously (5,15). We purchased a fiber-free diet (per 950 g) consisting of casein (250 g), corn starch (482.5 g), sucrose (100 g), soybean oil (70 g), mineral mix (35 g), vitamin mix (10 g), and choline bitartrate (2.5 g) from Unifaith Inc. (Seoul, Korea). For the preparation of AGP diets, we added 2 g AGP and 48 g corn starch to 950 g of the fiber-free diet to produce 1 kg of a 100 mg/kg/d AGP diet. For the preparation of the basal diet, 50 g of corn starch was added to 950 g of the fiber-free diet. Rats were randomly assigned to the sham, control, and AGP-treatment groups, and acclimatized for 3 days with the basal diet. In the AGP-treatment group, the rats (300 g) were fed with 15 g/d of the AGP diet, which is equivalent to a dose of 100 mg AGP/kg/d for 3 days before occlusion. After the rats consumed all the AGP diet food, more basal diet food was provided *ad libitum*. In the sham and control groups, the rats only received the basal diet.

Ischemia/reperfusion (IR) experiments

IR injury in male SD rats (~300 g) was induced by ligation of the left anterior descending coronary artery (LAD), as described previously (5,15). Rats were anesthetized with intramuscular injections of ketamine (100 mg/kg) and xylazine (5 mg/kg), intubated, and ventilated with air. The heart was exposed through a left thoracic incision, and the LAD was ligated about 5 mm down from aortic origin by passing a 5-0 Prolene suture (BV-1, Ethicon, Bridgewater, NJ, USA) around the LAD and double knotting the suture. Whether the LAD was occluded was confirmed by the left ventricular (LV) wall turning pale in color. The LAD of rats in the control and AGP-treated groups were ligated for 30 min and then reperfused for 3 h. The LAD of rats in the sham group underwent the same experimental procedures as those in the control group but without ligation.

Assessments of infarct size

The infarct size was determined by 2,3,5-triphenyltetrazolium chloride (TTC) staining as described previously (5,15). Briefly, 1 mL of 1.0% Evans blue (Sigma-Aldrich Co., St. Louis, MO, USA) was infused through the jugular vein after the LAD was religated, from which the area at risk (AAR) was defined as the area that was not infiltrated by Evans Blue. The heart was then harvested and excised into 4 pieces about 3 mm thick. The pieces of heart were stained in 2% TTC at 37°C for 10 min, from which infarct area (IA) was defined as the area that was not stained with TTC. Infarct size (IS) and risk size (RS) were defined as the percentages of the IA and AAR, and the AAR and the LV area (LVA), respectively. The border zone area (BZA) was defined as the region in which IA was excluded from AAR. AAR and IA were determined by computerized planimetry using ImageJ software (version 1.47, National Institutes of Health, Bethesda, MD, USA).

Terminal deoxynucleotidyltransferase-mediated dUTP nick-end labeling (TUNEL) staining

TUNEL staining (Millipore Corporation, Billerica, MA, USA) was performed to assess the extent of DNA nicking, as previously described (5,15). Briefly, the TUNEL-positive nuclei in the deparaffinized sections were assessed through incubation with terminal deoxynucleotidyl transferase and 5-bromo-2'-deoxyuridine-5'-triphosphate. Nuclei were identified by methyl green staining. To determine the percentage of apoptotic cells, micrographs of TUNEL-positive nuclei and methyl green-

stained nuclei were captured using an Olympus microscope (Olympus Corporation, Tokyo, Japan) and counted using ImageJ from 10 to 20 random fields at ×400 magnification. The ratio of the TUNEL-positive nuclei to total nuclei was presented.

Western blotting

To examine the protein levels of Bcl-2 and Bax, and phosphorylation of MAPK [p38, JNK, and extracellular signal-regulated kinases (ERK)], Western blotting was performed and the blots were analyzed as previously described (5,15). Briefly, heart proteins were extracted using radioimmunoprecipitation assay buffer (Cell Signaling Technology, Beverly, MA, USA) containing a protease inhibitor and phosphatase inhibitor cocktail (Roche, Mannheim, Germany). The proteins were separated on 10 or 15% sodium dodecyl sulfate-polyacrylamide gels according to the molecular weight of target proteins, and subsequently transferred to polyvinylidene fluoride membranes (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The membranes were blocked at room temperature with 5% non-fat skim milk for 1 h prior to incubation with the following primary antibodies: anti-Bcl-2 pAb (Santa Cruz Biotechnology, Dallas, TX, USA), anti-Bax (Santa Cruz Biotechnology), anti-phospho-p38 MAPKs mAb (pp38, Cell Signaling Technology), anti-p38 MAPKs pAb (p38, Cell signaling Technology), anti-phospho-JNK mAb (pJNK, Cell Signaling Technology), anti-JNK pAb (JNK, Santa Cruz Biotechnology), anti-phospho-ERK1/2 mAb (p-ERK1/2, Cell Signaling Technology), anti-ERK1/2 mAb (ERK1/2, Cell Signaling Technology), and anti-ERK1 pAb (ERK1, Santa Cruz Biotechnology). The membranes were incubated with horseradish peroxidase-labeled secondary antibodies (1:2,000, Enzo, Farmingdale, NY, USA) and developed with Amersham enhanced chemiluminescence prime Western blotting detection reagent (GE Healthcare, Little Chalofont, UK) using a ChemiDoc XRS Gel Imager (Bio-Rad Laboratories, Inc.). Protein bands were quantified using Image Lab software (version 5.1, Bio-Rad Laboratories, Inc.). ERK was employed as a loading control.

Statistical analysis

Values were expressed as mean±standard error of mean (SEM). Statistical analyses were performed using the SPSS software (version 19, IBM SPSS Statistics, Armonk, NY, USA). A one-way ANOVA followed by Tukey honestly significant difference *post hoc* test was used to compare changes in Bax/Bcl-2 ratios, and amounts of phosphorylated MAPK. Comparison of infarct size and amount of cellular apoptosis were analyzed by Student's *t*-test. Differences between the control and AGP-treated groups were considered statistically significant at $P<0.05$.

RESULTS AND DISCUSSION

Isolation and characterization of AGP

AGP was isolated from all-purpose wheat flour through hot ethanol extraction to remove proteins, water extraction to generate water soluble material, stepwise ethanol extraction to generate crude AGP, and fractional precipitation by ammonium sulfate to produce AGP at approximately 0.15% (w/w) yield (Fig. 1). GPC was used to analyze the purity and size of the isolated AGP (Fig. 2A). One major peak and one minor peak were observed, indicating that the AGP was nearly pure. The average molecular weight of the isolated AGP was approximately 15,500, similar to those previously reported, ranging from 20,000 to 25,000 (16-18). Weight percentage (wt%) of neutral monosaccharides analysis showed that the isolated AGP was composed of two major monosaccharides (57.7% galactose and 40.0% arabinose), and other minor monosaccharides (0.8% xylose, 1.2% glucose, and 0.3%

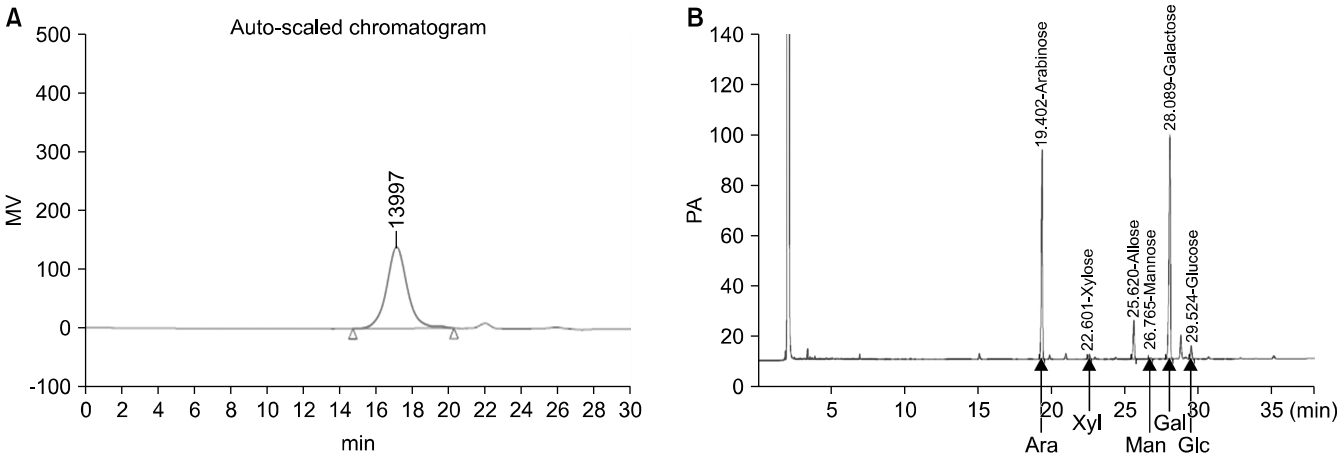

Fig. 2. Characterization of purified arabinogalactan-peptide (AGP). (A) Confirmation of purified AGP by gel permeation chromatography. A major single peak indicates that AGP is close to being pure. (B) Representative gas chromatography spectrum of the neutral monosaccharides of purified AGP. Ara, arabinose; Xyl, xylose; Man, mannose; Gal, galactose; Glc, glucose.

mannose) (Fig. 2B). The ratio of galactose to arabinose in the isolated AGP was approximately 1.5 (60:40), which is similar to ratios previously reported (14,17). The results indicate that nearly pure, authentic AGP was generated through multiple isolation steps.

Effect of AGP supplementation on infarct size in a rat IR model

To investigate whether AGP could protect against myocardial injury *in vivo* during the postocclusion steps of CHD, we used a rat IR model that mimics the opening of occluded arteries through reperfusion therapy in a clinical setting, such as during percutaneous coronary angioplasty (19). In the IR model, rats underwent 30 min ischemia through ligation of the LAD followed by 3 h reperfusion through release of the ligation after the rats were fed diets supplemented with 100 mg/kg/d AGP for 3 days. We chose the dose of 100 mg/kg/d based on our previous study demonstrating that AGP supplemented at 100 mg/kg/d was sufficient to reduce myocardial injury in the same rat model of myocardial infarction (13). Then we examined IS as a percentage of IA to AAR, which represents the degree of cell death in the AAR. The IA and AAR were determined by staining with TTC and Evans Blue dye staining, respectively. A schematic diagram of infarct size assessments is shown in Fig. 3A. A representative heart section indicating the AAR and IA, and LVA for the control and AGP-treated groups, respectively, is presented in Fig. 3B. These results show that supplementation with 100 mg/kg/d AGP significantly reduced the infarct size in rats compared with those in the control group (39.0±3.5% versus 54.5±1.1%, $P<0.05$) (Fig. 3C). To confirm the reliability of the results, we determined the risk size (RS), a percentage of AAR to LVA, which represents the occluded region in the LV. The RS in the AGP-treated group was not significantly different from that in the control group ($P>0.05$) (Fig. 3C), indicating the surgical procedures used to ligate the LAD were reliable. Similar efficacy was observed in our previous study (13). These findings indicate that AGP intake protects against IR injury during the postocclusion steps.

Effect of AGP intake on apoptosis *in vivo*

In our previous study, supplementation of hot-water extract of ground wheat protected against myocardial injury by inhibiting apoptosis (13). Therefore, we examined whether AGP supplementation could inhibit apoptotic cascades. We used TUNEL assays to measure the amount of DNA nicks in cells as a biomarker for apoptotic cells since DNA nicking occurs downstream in the apoptotic cascade (20). Apoptotic cells in the IA and BZ (AGP dose 100 mg/kg/d) were assessed separately because the amounts of apoptotic events in the two regions may vary (4,21) (Fig. 4A). We quantitatively determined the de-

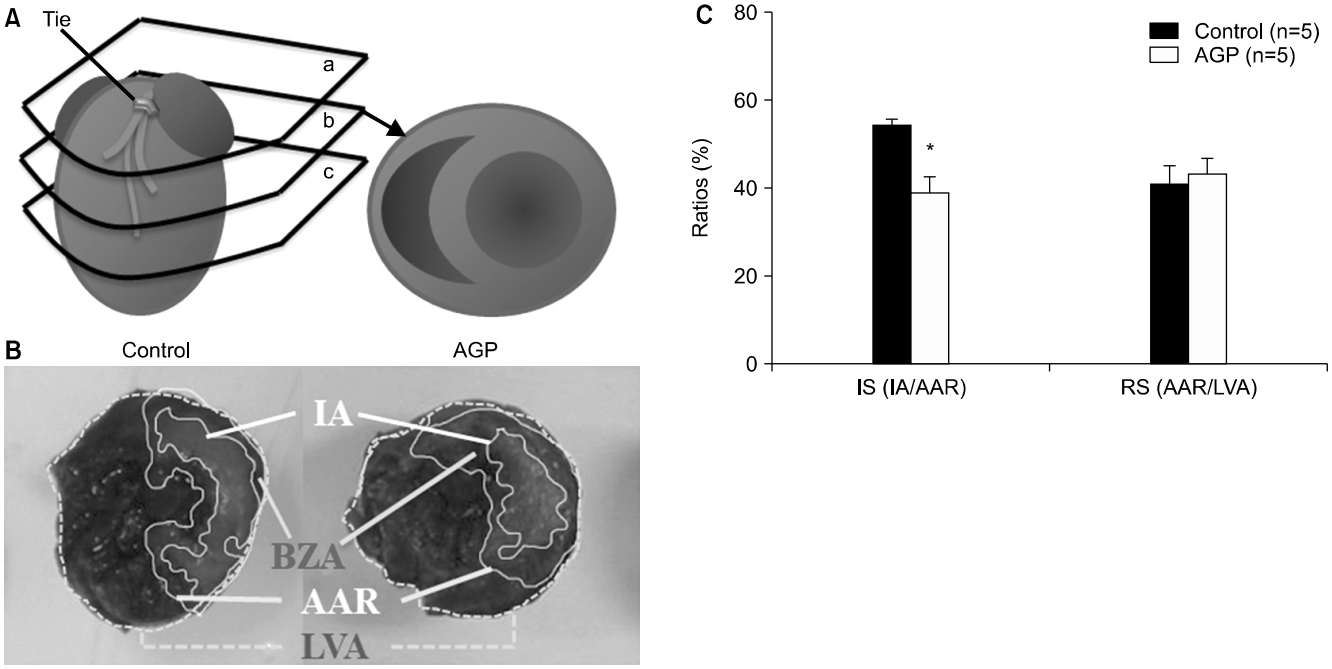

Fig. 3. Effect of arabinogalactan-peptide (AGP) supplementation on infarct size in rat IR models. (A) A schematic diagram of infarct size assessments. After IR experiments, the heart was harvested and excised into 4 pieces about 3 mm thick, and the pieces were stained through 2,3,5-triphenyltetrazolium chloride (TTC). (B) Representative heart sections [left ventricular area (LVA)] demarcating the area at risk (AAR) and infarct area (IA) for the control and AGP-treated groups, respectively. AAR and IA were defined as the area with Evans blue not infiltrated and the area with TTC not stained, respectively. In addition, the border zone (BZ) was defined as the region where IA is excluded from AAR. (C) Effect of AGP supplementation on infarct size. Infarct size (IS) and risk size (RS) were defined as the percentages of the IA and AAR, and the AAR and LVA, respectively. Values are expressed as means±SEM. $^*P<0.05$ compared with control group.

gree of apoptosis by calculating the ratio of TUNEL-positive cells to methyl green-stained cells, which reflect apoptotic cells and total cells, respectively, in randomly selected sections. The ratio of apoptotic cells to total cells in the BZ was significantly reduced in the AGP-treated group compared with the control group (7.8±1.1 versus 17.0±2.9, $P<0.05$) (Fig. 4B). These findings indicate that AGP supplementation inhibits apoptosis.

Effect of AGP supplementation on Bcl-2 and Bax levels

To investigate upstream of DNA nicking, the protein levels of anti-apoptotic Bcl-2 and pro-apoptotic Bax in the AAR were measured by immunoblotting (22). Representative results are shown in Fig. 5A. The Bax/Bcl-2 ratio, which determines cell fate, was significantly decreased in the AGP-treated group compared with the control group (0.52±0.15 versus 1.00±0.07, $P<0.05$) (Fig. 5B). These results indicate that AGP intake modulates Bcl-2 and Bax expression to inhibit apoptosis.

Effect of AGP supplementation on p38 and pJNK phosphorylation in MAPK pathway

Furthermore, we examined MAPK phosphorylation through immunoblotting since MAPK is located upstream of Bcl-2 and Bax in the apoptotic cascade in MI (7). Representative Western blots are presented in Fig. 6A. In the quantitative analysis, pp38/p38 and pJNK/JNK ratios were significantly reduced in the AGP-treated group compared with the control group (0.77±0.01 versus 1.00±0.05 for pp38/p38 and 0.38±0.14 versus 1.00±0.09% for pJNK/JNK, respectively, $P<0.05$) (Fig. 6B). These results reveal that AGP intake inhibits phosphorylation of p38 and JNK.

This study demonstrated that supplementation of AGP isolated from wheat flour inhibits apoptosis by decreasing phosphorylation of p38 and JNK, decreasing the Bax/Bcl-2 ratio and, subsequently, inhibiting apoptosis, resulting in a reduced infarct size in the rat IR model (Fig. 7). Similar observations were reported for larch arabinogalactan, which consists of arabinose and galactose, but

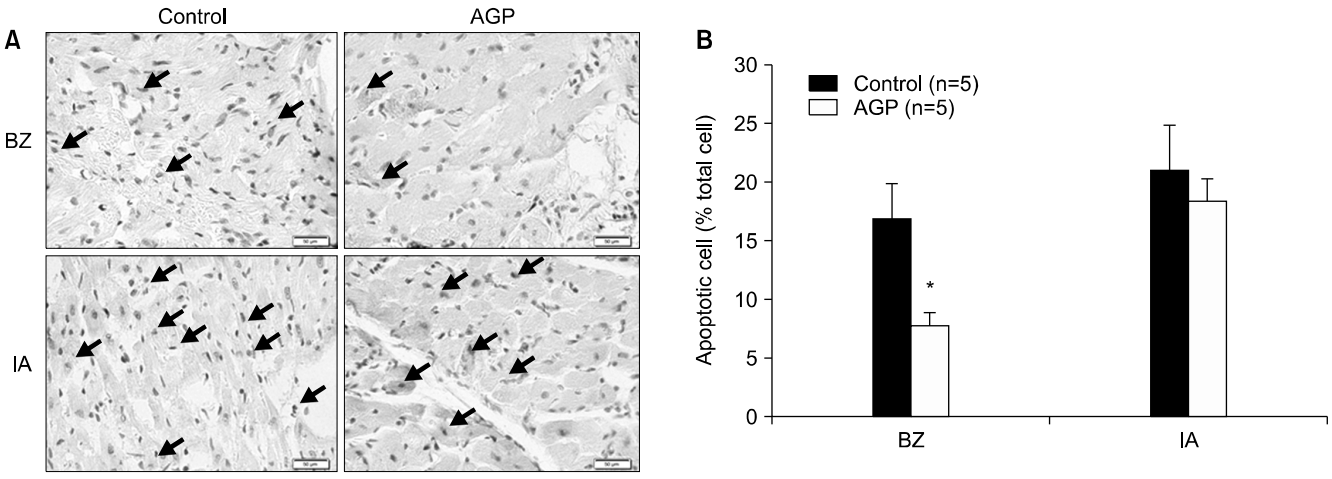

Fig. 4. Effect of arabinogalactan-peptide (AGP) supplementation on apoptosis *in vivo*. (A) Photomicrographs of terminal deoxynucleotidyltransferase-mediated dUTP nick-end labeling (TUNEL) staining (×400) of myocardial tissue sections for the control and AGP-treated groups taken from the border zone (BZ) and infarct area (IA). (B) Quantitative analysis of TUNEL-positive cells. The ratios of the TUNEL-positive cells to the total cells in BZ and IA are shown. Values are expressed as means±SEM. *$P<0.05$ compared with control group.

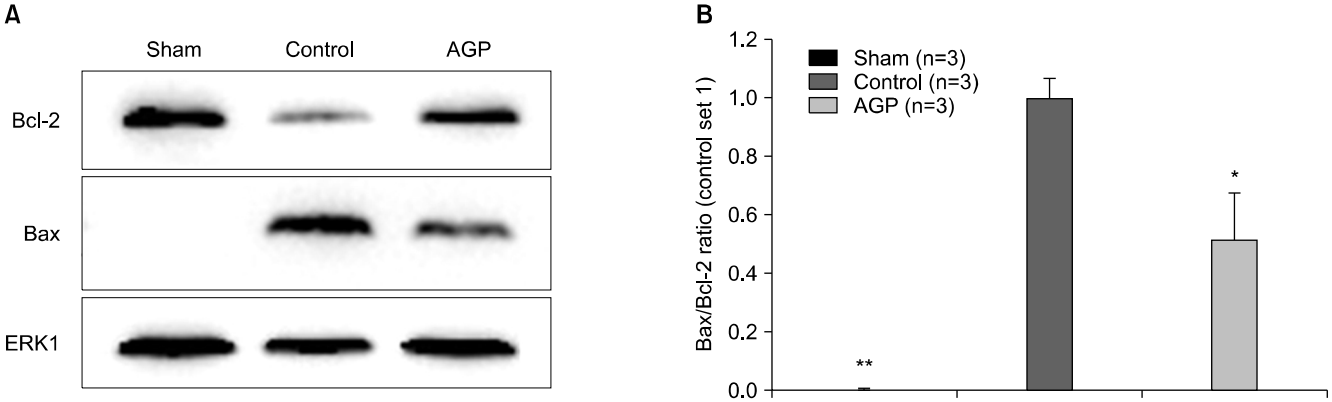

Fig. 5. Effect of arabinogalactan-peptide (AGP) supplementation on the B-cell lymphoma 2 (Bcl-2) and Bcl-2-associated X protein (Bax) levels. (A) Bcl-2 and Bax levels in the area at risk, as measured by Western blotting. (B) The levels of Bax/Bcl-2 ratio assessed by setting that of the control group to 1. Values are expressed as means±SEM. *$P<0.05$ and **$P<0.01$ vs. control group.

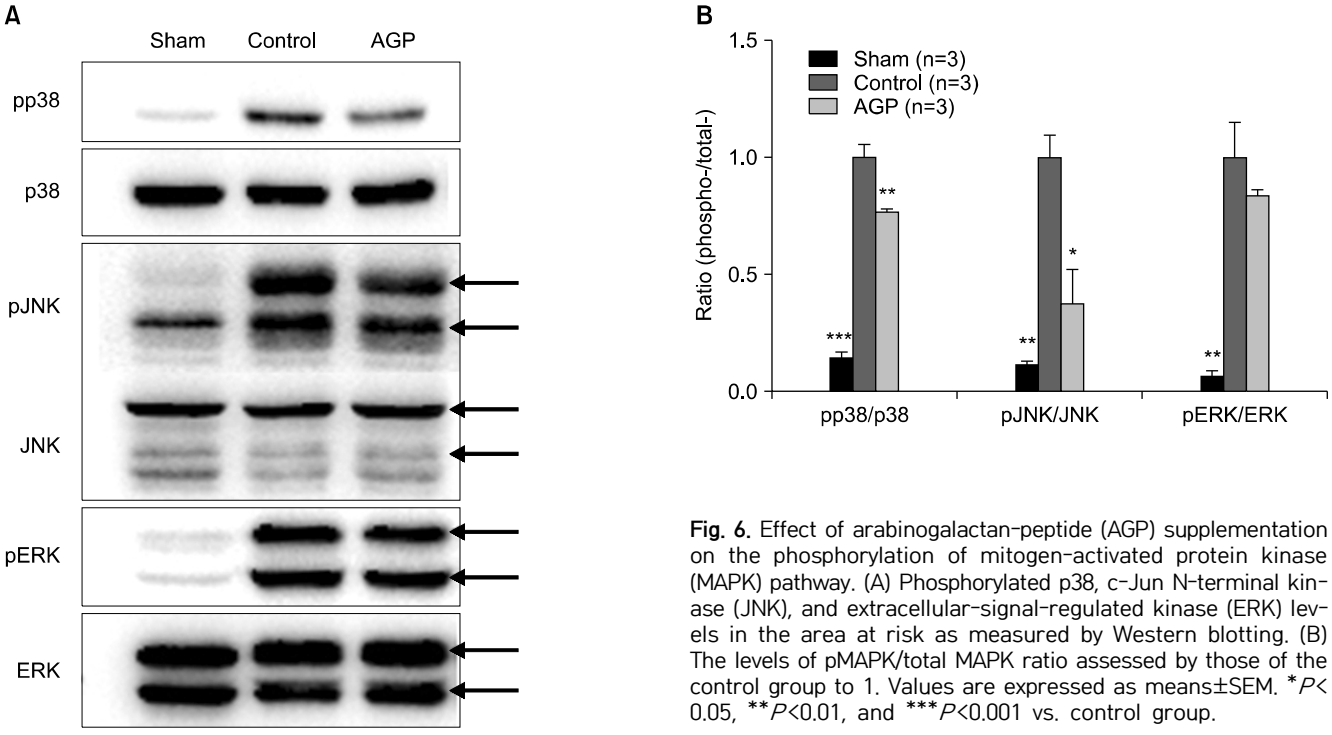

Fig. 6. Effect of arabinogalactan-peptide (AGP) supplementation on the phosphorylation of mitogen-activated protein kinase (MAPK) pathway. (A) Phosphorylated p38, c-Jun N-terminal kinase (JNK), and extracellular-signal-regulated kinase (ERK) levels in the area at risk as measured by Western blotting. (B) The levels of pMAPK/total MAPK ratio assessed by those of the control group to 1. Values are expressed as means±SEM. $^{*}P<0.05$, $^{**}P<0.01$, and $^{***}P<0.001$ vs. control group.

not peptides (6), which supports the idea that polysaccharides consisting of arabinose protect against myocardial injury. Our findings are supported by previous studies. First, myocardial IR activated p38 and JNK through phosphorylation (7), and inhibited p38 (23) and JNK phosphorylation (24) to protect against myocardial injury. Second, inhibition of p38 up-regulated Bcl-2, leading to inhibition of apoptosis, assessed through TUNEL assays (25).

Traditionally, reduction of CHD risk through consumption of dietary fiber such as AGP is thought to be associated with reduction of the risk factors such as dyslipidemia and hypertension at the preocclusion steps, which contribute to delayed narrowing and subsequent occlusion of the coronary arteries (12). In this study, we showed that consumption of AGP as a constituent of wheat can also reduce CHD risk at the postocclusion steps through reducing myocardial injury by inhibiting apoptosis, even after the coronary arteries are occluded.

In conclusion, consumption of AGP through wheat varieties containing high levels of AGP can be a plausible strategy to prevent CHD events by both inhibiting the postocclusion and preocclusion steps.

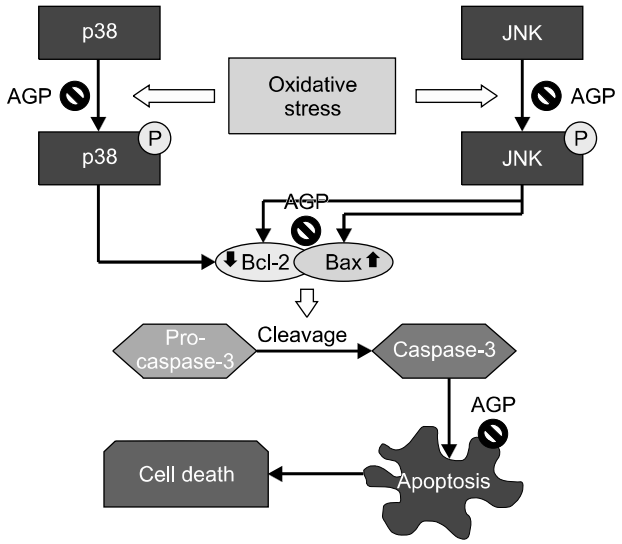

Fig. 7. A proposed mechanism for the role of arabinogalactan-peptide (AGP) supplementation on apoptosis. AGP supplementation inhibited apoptosis induced by oxidative stress through reducing p38 and JNK phosphorylation, reducing the Bcl-2-associated X protein (Bax)/B-cell lymphoma 2 (Bcl-2) ratio, and inhibiting generation of caspase-3 from procaspase-3.

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to M-J.H.

AUTHOR DISCLOSURE STATEMENT

The authors declare no conflict of interest.

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