Fatty Acids Ameliorate Doxorubicin-Induced Intracellular Ca\(^{2+}\) Increase and Apoptosis in Rat Cardiomyocytes

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Doxorubicin (Dox) is a highly effective anticancer drug but exhibits cumulative dose-dependent cardiomyopathy. In this study, we investigated effects of Magnolia seed extract (MagS) on the Dox-induced cardiotoxicity. The results showed that MagS significantly reduces doxorubicin (Dox)-induced increase in intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_{i}\)), generation of reactive oxygen species (ROS), and apoptosis in rat cardiomyocytes. Analyses of the bioactive compounds in MagS by thin layer chromatography and gas chromatography/mass spectroscopy revealed that bioactive compounds in MagS are linoleic acid, oleic acid, and palmitic acid. All three fatty acids were able to inhibit the Dox-induced increase in [Ca\(^{2+}\)]\(_{i}\), ROS generation, and apoptosis with a similar potency. Efficacy of MagS was examined in \textit{in vivo} using a murine Dox-induced cardiomyopathy model. Dox (12 mg/kg, intravenously) was administered to mice and treated with the MagS (2 mg/kg/d, intraperitoneally) or saline for three weeks. Dox-treated mice showed structural disarray in heart tissue, including lymphocyte infiltration and loss of body weight. In contrast, treatment of the MagS substantially attenuated the Dox-induced cardiac damages including the loss of body weight. These results indicate that fatty acids in MagS and other seeds may ameliorate cardiotoxicity of the anticancer drug.

**Key words** doxorubicin; fatty acid; cardiotoxicity; Ca\(^{2+}\) signaling; apoptosis; Magnolia

**MATERIALS AND METHODS**

**Extraction of Plant Material** Dried Magnolia seeds (300 g) were ground using a food processor and extracted with 11 of ethanol for overnight at room temperature. The solvent of Magnolia seed extract (MagS) was evaporated using a rotary evaporator at a maximum temperature of 45 °C. The amount of dried MagS was 62 g. The MagS was dissolved in ethanol (100 mg/ml).

**Isolation of Compounds** The MagS was separated by thin-layer chromatography (TLC) on silica gel GF254 using the mobile phase, chloroform : n-hexane : 2-propanol = 30 : 50 : 20. Ten bands from top to bottom, which were visualized under visible and UV lights, were scraped. Absorbed substances were extracted with absolute ethanol, and the extracts were obtained by centrifugation and dried using SpeedVac (AES1010, Savant, NY, U.S.A.) without heating. The dried compounds were weighed and dissolved in a small volume of absolute ethanol and kept at −20 °C until use.

**GC/MS Analysis** Experiments were carried out on a HP-6890 GC system connected to a HP-5973 mass selective detector fitted with a DB-5MS fused-silica capillary column (30 m×0.25 mm i.d., 0.25 μm film; SGE). Conditions were as follows: inlet pressure, 77.1 kPa; 8.7 ml He min\(^{-1}\); injection volume, 1 μl; transfer line, 250 °C; electron energy, 70 eV. The GC was programmed as follows: 5 min at 70 °C, increasing at 10 °C min\(^{-1}\) to 320 °C, operated in splitless mode (60-s valve time); He carrier gas at 1 ml min\(^{-1}\). Identification of compounds was performed by comparison of mass spectra to the Wiley-6 Library and the Essential Oils Library (Massfinder), by comparison with synthetic standards or retention-index data from the literature.\(^{11–14}\) All composi-

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tions were expressed as percentage (%) by area of total fatty acids.

**Preparation of Rat Cardiomyocytes** Cardiomyocytes were isolated from Sprague–Dawley rats, weighing 150—200 g, by the method as described previously. Briefly, hearts were rapidly excised, cannulated, and subjected to retrograde perfusion on a Langendorff apparatus at 37 °C with Ca2+-free Krebs–Hanseleit (KH) buffer (10 mm HEPES, 118 mm NaCl, 4.7 mm KCl, 1.2 mm MgSO4, 1.2 mm KH2PO4, 25 mm NaHCO3, 10 mm pyruvate, 11 mm dextrose, and 1 mm CaCl2, pH 7.3) for 5 min and then with KH buffer containing 10 mm of 2.3-butanedione monoxime (BDM), 5 mm taurine, 0.075% collagenase Type II, and 0.08 mg/ml protease Type IV and X for 7 min, and washed with KH buffer containing 0.2 mm Ca2+. The left ventricle was removed, chopped into small pieces, and incubated in a glass conical flask at 37 °C for 10 min with shaking. The undigested tissue was removed through a 200 μm mesh nylon filter. The Ca2+ concentration in the cell preparation was gradually increased up to 1 mm.

Isolated myocytes were pelleted by centrifugation at 60×g for 2 min at room temperature and resuspended in a stabilizing buffer (pH 7.4) containing 20 mm HEPES, 137 mm NaCl, 4.9 mm KCl, 1.2 mm MgSO4, 15 mm glucose, and 10 mm BDM. The cell preparation was kept in the stabilizing buffer containing 1% bovine serum albumin at room temperature about 1.5 h and then was washed three times with minimum essential medium (MEM) at 37 °C. Cardiomyocytes were seeded on the confocal dish coated with collagen (50 μg/ml) and incubated in the humidified 5% CO2 incubator for 20 min prior to addition of the substrate (20 μM BSA, 2 mm L-carnitine, 5 mm creatine, 5 mm taurine, 2 mm pyruvate, 100 IU/ml penicillin, and 100 μg/ml streptomycin). For the experiments, the cells were pretreated with various drugs at 37 °C for 10 min prior to addition of doxorubicin (Dox) (3 μM). At the indicated time point, the cells were washed with ice-cold PBS (140 mm NaCl, 2.7 mm KCl, and 10 mm KH2PO4/K2HPO4, pH 7.5). Excess PBS was removed by aspiration, and the cells were immediately frozen at −80 °C after addition of 500 μl of a lysis buffer (10 mm Tris–HCl, 10 mm KH2PO4/K2HPO4, pH 7.5, 130 mm NaCl, 1% Triton X-100, and 10 mm sodium pyrophosphatase). The cells were harvested, homogenized, and centrifuged at 15000 rpm at 4 °C for 30 min. Supernatants were collected, and protein concentrations were determined as described above. For in vivo experiment, hearts of Dox or drugs treated mice were rapidly frozen in liquid nitrogen. The tissues were homogenized with a grinder in cold lysis buffer (10 mm Tris–HCl, pH 8.0, 0.32% sucrose, 5 mm EDTA, 1 mm PMSF, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 2 mM DTT) and solubilized with lysis buffer containing 1% Triton X-100 at 4 °C for 1 h. After centrifugation, supernatant was collected and protein concentration was determined with a Bradford protein assay system (Bio-Rad). Samples (100 μg protein) were incubated with the substrate (20 μM, final concentration) at 37 °C for 1 h. Fluorescence of 7-amino-4-methylcoumarin liberated from the fluorogenic substrate was determined at 380 nm excitation and 460 nm emission using a fluorescence photometer, Spectra Max Gemini (Molecular Devices, Sunnyvale, CA, U.S.A.).

**Measurement of Caspase-3 Activity** Enzyme activity was measured using fluorogenic substrate, N-acetyl Asp-Glu-Val-Asp-7-amino-methylcoumarin (Ac-DEVD-AMC) using a method provided by the manufacturer (BD Biosciences). Cardiomyocytes were attached to collagen-coated plates at 37 °C for 10 min. After removing unattached cells, the cells were further incubated at 37 °C for 2 h in M199 medium (Gibco, Invitrogen, Grand Island, NY, U.S.A.) supplemented with 2 mg/ml BSA, 2 mm L-carnitine, 5 mm creatine, 5 mm taurine, 2 mm pyruvate, 100 IU/ml penicillin, and 100 μg/ml streptomycin. For the experiments, the cells were pretreated with various drugs at 37 °C for 20 min prior to addition of doxorubicin (Dox) (3 μM). At the indicated time point, the cells were washed with ice-cold PBS (140 mm NaCl, 2.7 mm KCl, and 10 mm KH2PO4/K2HPO4, pH 7.5). Excess PBS was removed by aspiration, and the cells were immediately frozen at −80 °C after addition of 500 μl of a lysis buffer (10 mm Tris–HCl, 10 mm KH2PO4/K2HPO4, pH 7.5, 130 mm NaCl, 1% Triton X-100, and 10 mm sodium pyrophosphatase). The cells were harvested, homogenized, and centrifuged at 15000 rpm at 4 °C for 30 min. Supernatants were collected, and protein concentrations were determined as described above. For in vivo experiment, hearts of Dox or drugs treated mice were rapidly frozen in liquid nitrogen. The tissues were homogenized with a grinder in cold lysis buffer (10 mm Tris–HCl, pH 8.0, 0.32% sucrose, 5 mm EDTA, 1 mm PMSF, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 2 mM DTI) and solubilized with lysis buffer containing 1% Triton X-100 at 4 °C for 1 h. After centrifugation, supernatant was collected and protein concentration was determined with a Bradford protein assay system (Bio-Rad). Samples (100 μg protein) were incubated with the substrate (20 μM, final concentration) at 37 °C for 1 h. Fluorescence of 7-amino-4-methylcoumarin liberated from the fluorogenic substrate was determined at 380 nm excitation and 460 nm emission using a fluorescence photometer, Spectra Max Gemini (Molecular Devices, Sunnyvale, CA, U.S.A.).

**Animal Experiment** Female BALB/c mice, 18 to 20 g weights, 6 to 8 weeks old, were obtained from the Orient Bio Inc. (SungNam, Republic of Korea), were acclimated to the facility for 5 d and fed and ad libitum with regular chow. Animal Research Committee of Chonbuk National University approved the animal study in accordance with the guidelines of the National Institutes of Health (NIH publication #85-23, 1985). Mice were divided into 4 groups. Group 1 received saline only and served: Control. Group 2 received an equivalent volume of saline 1 d after the administration of Dox (12 mg/kg, IV): Dox only. Group 3 received MagS (2 mg/kg, IP) 1 d after administration of Dox (12 mg/kg, IV): Dox+
MagS. Group 4 received MagS (2 mg/kg, IP) 1 d after administration of an equivalent volume of saline: MagS only. Mouse was maintained for 3 weeks and sacrificed for analysis.

**Histology** Mice were anesthetized with diethyl ether and a chest region was incised. Hearts were then removed from the mice and fixed overnight in a cold 10% formalin solution. Fixed tissues were processed routinely for paraffin embedding, and 5 μm sections were used for hematoxylin-eosine staining. Stained morphology was analyzed using a microscope (Leica, Wetzlar, Germany). The extent of lymphocyte accumulation was expressed on a semiquantitative rating scale ranging from 0 (no accumulation of lymphocyte) to 3 (lymphocyte accumulation present at least of 5 sites).

**Statistical Analysis** Data are expressed as mean±S.E.M. Statistical comparisons were performed using one-way ANOVA. Significant differences between groups were determined using the unpaired Student’s t test. Statistical significance was set at $p<0.05$.

**RESULTS**

**MagS Inhibits Dox-Induced $[\text{Ca}^{2+}]_i$ Increase in Rat Cardiomyocytes** As previously reported,7) treatment of rat cardiomyocytes with Dox slowly increased $[\text{Ca}^{2+}]_i$, beginning to increase at approximately 60—70 min after Dox treatment and reached plateau at approximately 90 min and sustained (Fig. 1A). Pretreatment of MagS inhibited Dox-induced $\text{Ca}^{2+}$ rise in a dose-dependent manner and the maximum inhibitory effect was observed with at 0.05 mg/ml (w/v) MagS (Fig. 1B). While MagS completely inhibited the Dox-induced $\text{Ca}^{2+}$ increase at 0.05 mg/ml (Fig. 1C), this concentration of MagS alone did not show any effect on $[\text{Ca}^{2+}]_i$ (Fig. 1D). Changes in $[\text{Ca}^{2+}]_i$ are summarized in Fig. 1E.

**MagS Inhibits Dox-Induced ROS Generation and Caspase-3 Activation** Dox is known to induce ROS generation and apoptosis.2,4,18) Consistent with previous observations, treatment of cardiomyocytes with Dox elevated ROS generation that was inhibited by pretreatment with MagS (0.05 mg/ml) (Fig. 2A). MagS also inhibited Dox-induced caspase-3 activation (Fig. 2B). MagS alone increased neither ROS level nor caspase-3 activity.

**Screening of Bioactive Compounds** Bioactive compounds in MagS were fractionated using preparative thin-layer chromatography. Visualized ten bands under visible and UV lights were scraped from thin-layer sheet. Acquired compounds were extracted with ethanol and then tested the ability of the compounds to inhibit the Dox-induced $\text{Ca}^{2+}$ increase. As shown in Fig. 3, the third fraction contained the bioactive components. Ability of the compounds in each fraction (0.01 mg/ml) to inhibit Dox-induced $\text{Ca}^{2+}$ raise observed at 90 min is summarized in Fig. 3M.

The third fraction that reduces Dox-induced $[\text{Ca}^{2+}]_i$ elevation in cardiomyocytes was analyzed by GC/MS methods to identify the molecules. Two major peaks were observed (Fig. 4A). The first peak with retention time of 17.291 min was n-hexadecanoic acid (15.708% as area %), a class of palmitic acid (Fig. 4B). The second peak overlapped with two components and the peak with retention time of 18.931 min was 9,12-octadecadienoic acid (39.684% as area %), a class of linoleic acid (Fig. 4C), and the other one (retention time, 18.971 min) was 9-octadecenoic acid (31.208% as area %), a class of oleic acid (Fig. 4D).

**Fatty Acids Prevent Dox-Induced Cardiotoxicity** The fatty acids obtained by GC/MS analysis were examined
Fig. 3. Comparison of Inhibitory Effect against Dox-Mediated Increase of $\left[\text{Ca}^{2+}\right]_{i}$ by Fractionated MagS

Rat cardiomyocytes were preincubated with MagS (0.05 mg/ml) or each fraction of MagS (0.01 mg/ml) at 37°C for 20 min, and then $\text{Ca}^{2+}$ response was observed in the presence of 3 μM Dox. (A) Dox only, (B) Dox+MagS, (C to L panel) Dox+fraction no. 1 to 10, (M) a direct comparison of the $\text{Ca}^{2+}$ level at 80 min. Data are presented as mean±S.E.M. from three independent experiments. *p<0.01, **p<0.05 versus Dox.

Fig. 4. Mass Spectra of Third Fraction Extracted from TLC Plate Separating MagS

The components in fraction 3 that exhibit bioactivity was analyzed by GC/MS. (A) Mass spectra of third fraction of MagS. (B, C, D) Mass spectra of major peak shown in panel A. Analyzed GC/MS data (top) arranged with standard mass profile of database (bottom). Commercial name, formula weight of identified fatty acid, and the structure of fatty acid with cleavage sites are described in inset.
whether these fatty acids exhibit detoxification ability to cardiomyocytes. Dox-induced Ca$^{2+}$ increase was significantly reduced by pretreatment with 10 mM linoleic acid, oleic acid, and palmitic acid (Fig. 5A). Dox-induced ROS generation was also reduced significantly by these three fatty acids (Fig. 5B). Dox-induced caspase-3 activation was significantly inhibited by the fatty acids (Fig. 5C). These results suggest that fatty acids in MagS ameliorate Dox-induced cardiotoxicity.

MagS Reduces Dox-Induced Cardiotoxicity in Mice

To further evaluate the in vitro results, we examined MagS in vivo using a mouse cardiomyopathy model induced by administration of Dox (Fig. 6). As expected administration of Dox resulted in cardiac tissue abnormality, showing lymphocyte infiltration and abnormal structure such as formation of vacuole and myofibrillar disarray (Fig. 6B). Upon treatment of Dox-treated mice with MagS, Dox-induced infiltration of lymphocytes and structural disarray of cardiac tissue were significantly reduced (Fig. 6C). MagS only showed no toxicity, showing similar results to control (Fig. 6D). MagS effects on Dox-induced cardiomyopathy including lymphocyte infiltration were summarized in Fig. 6E. In addition, treatment with Dox prevented gaining of body weights, losing body weight by 18.5 ± 4.5% at 3 weeks later (Fig. 6F). In case of Dox+MagS group, a significant loss of body weight was not observed. However, the body weight of mice treated with MagS only group was increased as the control mice did. To assess the incidence apoptotic cell death in cardiac tissues, we determined the caspase-3 activity in the extracts of hearts from Dox-treated mice and MagS only group. The enzyme activity in the extracts of hearts from Dox+MagS group was increased significantly compared to that in the control. The enzyme activity in the extracts of hearts from Dox-treated mice was increased significantly compared to that in the control and the Dox-induced increase of the caspase-3 activity was reduced significantly by treatment with MagS (Fig. 6G).

Fig. 5. Effect of Linoleic Acid, Oleic Acid and Palmitic Acid against Dox-Induced Toxicity in Rat Cardiomyocytes

A direct comparison of the Ca$^{2+}$ level at 80 min (A), ROS generation (B), and caspase-3 activation (C) with 1 μM, 10 μM, and 20 μM of fatty acids. These data are presented mean ± S.E.M. from three independent experiments in duplicate. *$p$ < 0.01 and **$p$ < 0.05; †$p$ < 0.01 and ††$p$ < 0.01 versus basal control (no drug treatment).

Fig. 6. MagS Reduces Cardiomyopathy and Decrease Body Weight in Mice Model

Hematoxylin-eosin staining of heart ventricle from BALB/c mice after treatment with or without Dox and MagS (A) Control mice, (B) Dox only, (C) Dox+MagS, (D) MagS only group. The infiltrating lymphocytes are observed in Dox only group while other groups are negative (black arrow). White scale bar=50 μm. (E) A relative score of the infiltrated lymphocyte compared from both group. (F) Effect of MagS against Dox-induced decrease of body weight. (G) Effect of MagS on caspase-3 activation by Dox administration. *$p$ < 0.01 and **$p$ < 0.05; †$p$ < 0.005 and ††$p$ < 0.01; †$p$ < 0.05 and ††$p$ < 0.05 versus basal control (no drug treatment).
DISCUSSION

Dox has been in clinical use as an anti-cancer agent for a wide range of malignant tumors. However, its use has been limited by a dose-related and irreversible cardiotoxicity.\(^{3,9}\) Dox-induced cardiotoxicity is known to be caused mainly by ROS generation. In addition, a disturbance of Ca\(^{2+}\) homeostasis by Dox is also implicated one of the cardiotoxic mechanisms.\(^{10}\) Therefore, controlling ROS generation and maintaining Ca\(^{2+}\) homeostasis seem to be an approach for the prevention of cardiotoxicity induced by Dox.

Bioactive components from root and/or stem bark of Magnolia are known to contain bioactive components such as honokiol and magnolol.\(^{16,20,21}\) In this study, we have investigated Magnolia seed extract to evaluate biological effects on Dox-induced cytotoxicity in \textit{in vitro} and \textit{in vivo}. Our results showed that MagS was able to inhibit Dox-induced increase in [Ca\(^{2+}\)], ROS generation, and apoptosis in rat cardiomyocytes. Analysis of MagS revealed that the bioactive components in MagS were n-hexadecanoic acid (a class of palmitic acid), 9,12-octadecadienoic acid (a class of linoleic acid), and 9-octadecenoic acid, (a class of oleic acid). These fatty acids were able to inhibit Dox-induced increase in [Ca\(^{2+}\)], ROS generation, and apoptosis in rat cardiomyocytes. Assessment of the ability of MagS in \textit{in vivo} revealed that Dox-induced cardiotoxicities such as infiltration of lymphocytes and disarray of cardiac muscle structure are significantly attenuated by treatment with MagS. In addition, several fatty acids have been shown to exhibit an anti-cancer activity by single or combination therapy with anthracyclins by increasing Dox cytotoxicity in human breast tumor cell line.\(^{21,23}\) Although anticancer activity of MagS is unknown, MagS did not block Dox-induced Ca\(^{2+}\) increase in HL60 (leukemia), MDA-MB-231 (breast cancer), B16 (melanoma), Panc-1 (pancreatic cancer) (data not shown).

Studies have demonstrated that some fatty acids can attenuate cardiovascular diseases. Thus, Kang and Leaf have reported that eicosapentaenoic acid (EPA; 20 : 5n-3) and docosahexaenoic acid (DHA; 22 : 6n-3) exert protective effects against cardiovascular diseases such as arrhythmias generated by post-infarction.\(^{24,25}\) The cardioprotective effect of these fatty acids is due to a reduction of ion channel activity by a direct interaction with the channel in plasma membranes. Brustein with the observations, EPA has also been shown to reduce voltage-gated L-type Ca\(^{2+}\) currents in adult and neonatal cardiomyocytes.\(^{20}\) Vitelli \textit{et al.} have suggested that DHA is capable of inhibiting Dox- or caffeine-induced release of Ca\(^{2+}\) from SR.\(^{25}\) In the present study, our results showed that linoleic acid, oleic acid, and palmitic acid in MagS extract completely blocked Dox-induced Ca\(^{2+}\) increase at micromolar concentrations. We have previously reported that Dox induces Ca\(^{2+}\) release from SR via opening of ryanodine receptor.\(^{4}\) In addition, studies have reported that Dox induces formation of vacuoles and disruption of myocardial architecture, infiltration of lymphocytes, and formation of vacuoles, including loss of body weight. All of these pathologic features were ameliorated by MagS.

In summary, fatty acids such as linoleic acid, oleic acid and palmitic acid in Magnolia seed extract exhibit protective effects on Dox-induced cardiotoxicities such as Ca\(^{2+}\) raise, ROS generation, and caspase-3 activation. Our results suggest that intake of fatty acids is recommendable for minimizing cardiac damage caused by Dox during cancer therapy.

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REFERENCES AND NOTES

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