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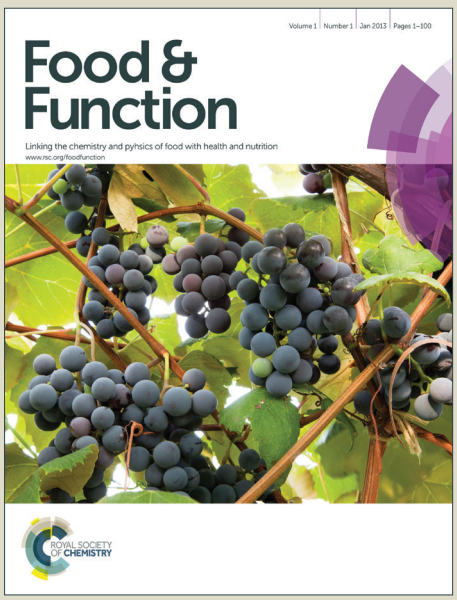

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Asiatic acid and maslinic acid protected heart via anti-glycative and anti-coagulatory activities in diabetic mice

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Running title: cardiac protection of asiatic acid and maslinic acid

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Abstract

Cardiac protective effects of asiatic acid (AA) and maslinic acid (MA) in diabetic mice were examined. These triterpenoids at 0.1 or 0.2% of the diet were supplied to diabetic mice for 12 weeks. AA or MA treatments decreased plasma glucose and HbA1c levels, creatine phosphokinase and lactate dehydrogenase activities in diabetic mice (p<0.05). AA or MA intake increased their deposit in heart; retained cardiac glutathione content; and reduced the production of reactive oxygen species, N^{ε}-(carboxymethyl)-lysine, pentosidine, methylglyoxal, interleukin-6, tumor necrosis factor-alpha and monocyte chemoattractant protein-1 in heart of diabetic mice (p<0.05). AA or MA intake lowered plasma von Willebrand factor and fibrinogen levels, and factor VII activity (p<0.05); also maintained circulating antithrombin-III and protein C activities (p<0.05). AA or MA treatments down-regulated cardiac expression of NADPH oxidase, aldose reductase, nuclear factor kappa B (NF-κB) p65 and p-p38; as well as reserved glyoxalase 1 expression (p<0.05). These two compounds only at 0.2% lowered cardiac expression of NF-κB p50, p-ERK1/2 and receptor of advanced glycation endproduct (p<0.05). These findings support that the supplement of these triterpenoids could protect heart under diabetic condition via attenuating glycative injury and coagulatory disorders.

Keywords: Diabetes; Glycation; Coagulation; Asiatic acid; Maslinic acid

Introduction

Coagulatory disorders often occur in diabetic patients with poor glycemic control.[1,2] The increase of coagulation factors such as factor VII (FVII) and fibrinogen; and/or the decrease of anti-coagulation factors such as protein C and antithrombin-III (AT-III) in circulation of diabetic patients caused hypercoagulability, which facilitated the development of cardio-vascular diseases like diabetic cardiomyopathy because heart is vulnerable to diabetes associated thrombosis.[3,4] Furthermore, excessive production of reactive oxygen species (ROS) under diabetic condition from the activation of NADPH oxidase acts as signaling element in thrombogenic cycle, and favors the progression of local or systemic thrombosis.[5,6] Thus, hemostatic imbalance warrants more attention in order to avoid the occurrence of diabetic cardiomyopathy.

Aldose reductase (AR) is the rate-limiting enzyme of polyol pathway and converts glucose to fructose.[7] Excessive glucose and fructose facilitate the formation of advanced glycation endproducts (AGEs) under diabetic condition. N^{ε}-(carboxymethyl)-lysine (CML) and pentosidine are predominant AGEs contributed to the progression of diabetic cardiomyopathy in diabetic patients.[8,9] On the other hand, glyoxalase 1 (GLO-1), the rate-limiting enzyme of glyoxalase system, detoxifies AGE's precursors such as methylglyoxal to less toxic products like D-lactate.[10] Thus, any agent with the ability to inhibit AR and/or enhance GLO-1 expression may decrease AGEs production and diminish glycative stress. Furthermore, AGEs upregulate their membrane-anchored receptor (RAGE), and AGE-RAGE interaction activates mitogen-activated protein kinase (MAPK) and nuclear factor kappa-B (NF-κB) signaling pathways,[11,12] which in turn accelerates the production of inflammatory factors like tumor necrosis factor (TNF)-alpha. Thus, the suppression on RAGE, MAPK and NF-κB pathways

may delay diabetic progression.

Asiatic acid (AA) and maslinic acid (MA) are pentacyclic triterpenes naturally occurring in many vegetables and fruits such as brown mustard (*Brassica juncea*), centella (*Centella asiatica* L.) and olive (*Olea europaea L.*).[13,14] Castellano *et al.*[15] indicated that oleanolic acid related pentacyclic triterpenes like MA provided beneficial effects against diabetes through regulating antioxidant enzymes and transduction pathways. Ramachandran *et al.*[16] reported that AA improved glycemic control and lipid metabolism in diabetic rats. Thus, AA and MA are potent anti-diabetic agents. However, it remains unknown whether AA or MA could protect heart against diabetic coagulatory and glycative disorders. Our previous study found that dietary intake of AA and MA increased their bioavailability in heart of mice.[14] Thus, it is highly possible that the presence of these triterpenes in heart could protect this organ against diabetes related damage.

The major purpose of our present study was to investigate the anti-glycative effects of AA and MA at various doses in heart of diabetic mice. The influence of these compounds upon protein expression of AR, GLO-1, NADPH oxidase, RAGE and MAPK in heart of diabetic mice was evaluated. Furthermore, the impact of these compounds upon coagulatory and anti-coagulatory factors was also examined. These results will enhance our understanding regarding the application of these triterpenes against diabetic cardiomyopathy.

Materials and Methods

Chemicals

Asiatic acid (AA, 98%) and maslinic acid (MA, 97.5%) were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA).

Animals and diets

Male Balb/cA mice, 3-week old, were obtained from National Laboratory Animal Center (National Science Council, Taipei City, Taiwan). To induce diabetes, mice with body weight at 23.7± 1.2 g were treated with a single i.v. dose (50 mg/kg) of streptozotocin dissolved in citrate buffer (pH 4.5) into the tail vein of mice after 12 hr fast. Blood glucose level was monitored on day 10 from the tail vein after 12 hr fast by using a one-touch blood glucose meter (Lifescan Inc., Milpitas, CA, USA). Mice with fasting blood glucose level ≥ 200 mg/dl were used for this study. Animal experiments were performed in accordance with protocols approved by the Animal Care Research Committee of China Medical University (102-36-N).

Experimental design

After diabetes was induced, mice were divided into five groups (10 mice per group): diabetic mice with 0, 0.1% AA, 0.2% AA, 0.1% MA, 0.2% MA. In addition, one group of non-diabetic mice was divided into three sub-groups, in which 0 (control), 0.2% AA or 0.2% MA was supplied. All mice had free access to feed and water at all times. Consumed water volume and feed were recorded. Body weight and plasma glucose level were measured weekly. After 12 weeks supplementation, mice were fasted for 12 hr, and killed with carbon dioxide. Blood and heart from each mouse were collected and weighted. Protein concentration of heart homogenate was determined by a commercial assay kit (Pierce Biotechnology Inc., Rockford, IL, USA) with bovine serum albumin as a standard.

Blood analyses

Plasma glucose level (mg/dl) was measured by a glucose kit (Sigma Chemical Co., St. Louis, MO, USA). Plasma HbA1c level was measured by a DCA 2000 analyzer (Bayer-Sankyo, Tokyo, Japan). Plasma insulin level (μg/l) was determined by an insulin

radioimmunoassay kit (Linco Research Inc., St. Charles, MO, USA). Plasma activities of lactate dehydrogenase (LDH), creatine phosphokinase (CPK), alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were measured by assay kits purchased from Randox Laboratories Ltd. (Crumlin, UK).

Determination of cardiac AA or MA content

The content of target compound in heart was analyzed by the method described in Gerbeth *et al.*[17] Cardiac homogenate, 100 μl, was mixed with glycyrrhetinic acid as an internal standard (10 μl of 2.0 μg/ml methanol solution), and followed by extracting with 1 ml ethyl acetate and centrifuging at 3500 xg for 10 min at 4°C. After evaporated by nitrogen, the residue was reconstituted in 100 μl of methanol and water, the mobile phase of HPLC. Identification and quantification was processed by an HPLC-MS system (Agilent Corp, Waldbronn, Germany), in which Agilent 1100 series HPLC equipped with a BDS RP-C18 column (100 mm × 4 mm, 3 μm, Thermo Electron, Bellafonte, PA, USA), and a diode array and a fluorescence detector were applied. An ion-trap mass spectrometer equipped with an electro-spray ionization source was coupled with HPLC, and a negative single ion mode was used for analysis. The limit of detection was 0.1 μg/g tissue. The relative standard deviations of precision and accuracy for test compound were 3.7 and 4.1%, respectively.

Measurement of coagulation and anti-coagulation factors

Blood samples were anti-coagulated using sodium citrate. vWF antigen level was measured by a rabbit anti-rat vWF polyclonal antibody (Dako, Glostrup, Denmark), and vWF level was expressed as relative percentage of normal pooled plasma. FVII activity was determined by a kit bought from Chromogenix Co. (Lexington, MA, USA), which was positively correlated to the production of p-nitroaniline, and the absorbance at 405 nm was

assayed. Plasma fibrinogen level (g/l) was measured using a commercial kit (Iatroset Fbg, Iatron Laboratory, Tokyo, Japan). PAI-1 activity (kU/l) was assayed by a kit purchased from Trinity Biotech plc. (Wicklow, Ireland). The activity of AT-III and protein C in plasma was measured by AT-III and protein C kits (Sigma Chemical Co., St. Louis, MO, USA), respectively. The activity of AT-III and protein C was expressed as a percentage of standard plasma.

Determination of CML, pentosidine, fructose, methylglyoxal and D-lactate levels

Cardiac tissue at 50 mg was homogenized and digested with proteinase K (1 mg/ml) for 3 hr at 37°C, and reaction was stopped by 2 mmol/l phenylmethylsulfonyl fluoride. CML was immunochemically determined with a competitive ELISA kit (Roche Diagnostics, Penzberg, Germany) using the CML-specific monoclonal antibody 4G9, and calibration with 6-(N-carboxymethylamino)caproic acid. Absorbance at 405 nm (reference 603 nm) was read in a microtiter ELISA plate reader (Bio-Rad, Hercules, CA, USA). Pentosidine level was analyzed by a HPLC equipped with a C18 reverse-phase column and a fluorescence detector (Waters, Tokyo, Japan) with an excitation and emission wavelength at 335 and 385 nm according to the method described by Miyata *et al.*[18] Briefly, sample was lyophilized and acid hydrolyzed in 500 µl 6 N HCl for 16 hr at 110°C in screw-cap tubes purged with nitrogen. After neutralization with NaOH, sample was used for HPLC measurement. In addition, 50 mg heart was homogenized with phosphate buffer containing U-[^{13}C]-sorbitol as an internal standard. After precipitating protein and centrifugation, the supernatant was lyophilized. The content of fructose in lyophilized sample was determined by liquid chromatography with tandem mass spectrometry, according to the method of Guerrant and Moss.[19] Methylglyoxal level was analyzed by a HPLC method.[20] Cardiac sample was derivatized with perchloric

acid and o-phenylenediamine, and followed by purification with solid-phase extraction on AccuBond extraction cartridges. After centrifugation at 5,000 xg for 20 min, supernatant was collected. Quantification of methylglyoxal was processed by a HPLC system equipped with a fluorescence detector and a RP-C18 column (5 μm particle size, 250 x 4 mm i.d.). D-lactate level was determined by an enzymatic assay kit purchased from Eton Bioscience (San Diego, CA, USA). Cardiac tissue at 25 mg was homogenized, and D-lactate was extracted by ethanol. After centrifugation at 10,000 xg for 10 min, supernatant was used for D-lactate measurement.

Measurement of ROS, glutathione (GSH), interleukin (IL)-6, TNF-alpha and monocyte chemoattractant protein (MCP)-1 levels

Cardiac tissue was homogenized with ice-cold phosphate buffer containing 0.05% Tween 20 and 1 mM EDTA. Intracellular ROS level was determined using an oxidation sensitive dye, 2',7'-dichlorofluorescein diacetate (DCFH-DA). Briefly, 500 μl homogenate was mixed with 500 μl of 2 mg/ml DCFH-DA for 30 min at 37°C. Fluorescence was measured at 488 nm excitation and 525 nm emission by a fluorescence plate reader. Results are expressed as relative fluorescence unit (RFU) per mg protein. GSH concentration was measured by a commercial colorimetric glutathione assay kit (OxisResearch, Portland, OR, USA). Levels of IL-6 and TNF-alpha were quantified using ELISA kits (R&D Systems, Minneapolis, MN, USA). MCP-1 level was assayed by a cytoscreen immunoassay kit (BioSource International, Camarillo, CA, USA).

Western blot analysis

Cardiac tissue, 40 mg, was homogenized in buffer containing 0.5% Triton X-100 and protease-inhibitor cocktail (1:1000, Sigma-Aldrich Chemical Co., St. Louis, MO, USA). This homogenate was further mixed with buffer (60 mmol/l Tris-HCl, 2% SDS and 2%

β-mercaptoethanol, pH 7.2), and boiled for 5 min. Sample at 40 μg protein was applied to 10% SDS-polyacrylamide gel electrophoresis, and transferred to a nitrocellulose membrane (Millipore, Bedford, MA, USA) for 1 hr. After blocking with a solution containing 5% nonfat milk for 1 hr, membrane was incubated with anti-RAGE (1:500), anti-AR, anti-SDH, anti-GLO-1, anti-GLO-2, anti-$p47^{phox}$, anti-$gp91^{phox}$, anti-NF-κB p65, anti-NF-κB p50 (1:1000) or anti-MAPK (1:2000) monoclonal antibody (Boehringer-Mannheim, Indianapolis, IN, USA) at 4ºC overnight, and followed by reacting with horseradish peroxidase-conjugated antibody for 3.5 hr at room temperature. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a loading control. The detected bands were quantified by an image analyzer (ATTO, Tokyo, Japan) and normalized against GAPDH.

Statistical analysis

All data were expressed as mean±standard deviation (SD). Statistical analysis was done using one-way analysis of variance, and post-hoc comparisons were carried out using Dunnett's t-test. Statistical significance is defined as $p < 0.05$.

Results

Effects of AA or MA upon diabetic characteristic.

For normal mice, AA or MA intake at 0.2% increased their deposit in heart, but did not affect any measurements. As shown in Table 1, diabetes lowered body weight, increased feed intake and water intake ($p<0.05$); however, AA or MA intake at two doses improved body weight loss and lowered feed intake; only at 0.2% decreased water intake ($p<0.05$). AA or MA intake at 0.2% increased cardiac content of these compounds in diabetic mice. Diabetes decreased plasma insulin level, increased plasma glucose and HbA1c levels, and CPK and LDH activities

(Table 2, $p<0.05$). AA or MA intake at both doses lowered plasma glucose and HbA1c levels, CPK and LDH activities ($p<0.05$); but plasma insulin level was restored by these compounds only at 0.2% ($p<0.05$). AA or MA intake did not affect ALT and AST levels ($p>0.05$).

Effects of AA or MA upon coagulatory and anti-coagulatory factors.

Diabetes caused the increase in plasma level or activity of vWF, FVII, fibrinogen and PAI-1 (Table 3, $p<0.05$). AA or MA intake at both doses decreased vWF level, and at 0.2% lowered fibrinogen level and FVII activity ($p<0.05$); but failed to alter PAI-1 activity ($p>0.05$). Diabetes reduced plasma AT-III and protein C activities ($p<0.05$). AA or MA intake retained AT-III activity, and only at 0.2% maintained protein C activity ($p<0.05$).

Effects of AA or MA upon oxidative and glycative factors.

Diabetes lowered GSH content; and increased ROS level in heart (Table 4, $p<0.05$); AA or MA intake at both doses retained cardiac GSH content and reduced cardiac ROS level ($p<0.05$). Diabetes also increased CML, pentosidine, fructose and methylglyoxal levels, as well as decreased D-lactate level in heart ($p<0.05$). AA or MA intake at test doses lowered cardiac levels of CML, pentosidine, fructose and methylglyoxal; and raised D-lactate generation ($p<0.05$). As shown in Fig. 1, diabetes up-regulated cardiac expression of $p47^{phox}$, $gp91^{phox}$, AR, SDH and RAGE; and down-regulated cardiac expression of GLO-1 and GLO-2 ($p<0.05$). AA or MA intake at two doses down-regulated $p47^{phox}$ and AR expression, and only at 0.2% reduced $gp91^{phox}$ and RAGE expression ($p<0.05$). AA or MA intake at test doses also reserved GLO-1 expression ($p<0.05$). Cardiac SDH and GLO-2 expression were not affected by either AA or MA ($p>0.05$).

Effects of AA or MA upon NF-κB and MAPK expression.

Diabetes increased cardiac release of MCP-1, IL-6 and TNF-alpha (Table 5, $p<0.05$). AA or

MA intake at both doses decreased these cytokines in heart (p<0.05). Diabetes enhanced cardiac expression of NF-κB and MAPK (Fig. 2, p<0.05). AA or MA intake at two doses down-regulated NF-κB p65 and p-p38 expression (p<0.05). These two compounds only at 0.2% lowered NF-κB p50 and p-ERK1/2 expression in heart (p<0.05).

Discussion

Our present study found that the intake of AA or MA resulted in their deposit in heart, benefited glycemic control and protected heart against diabetes induced hyper-coagulatory and glycative stress. Moreover, AA and MA could regulate cardiac NADPH oxidase, polyol, NF-κB and MAPK pathways, which in turn alleviated oxidative and inflammatory injury in heart of diabetic mice. These findings suggest that these two triterpenoids could provide multiple protections for heart against diabetes.

Hypercoagulability is an important contributor for the development of vascular complications for diabetic patients.[21] vWF is involved in platelet adhesion and aggregation.[22] FVII is the first enzyme in blood coagulation system for triggering the clotting cascade.[23] Fibrinogen is a precursor in fibrin formation and a cofactor in platelet aggregation; and PAI-1 is the primary physiologic inhibitor of fibrinolysis.[24] Thus, the elevated circulating level or activity of vWF, FVII, fibrinogen and PAI-1 in those diabetic mice revealed a predominance of coagulation, which obviously favored the progression of thrombosis. However, our data indicated that AA or MA supplement at 0.1 and/or 0.2% lowered vWF and fibrinogen levels, and FVII activity, which contributed to attenuate coagulatory disorder. On the other hand, AT-III and protein C are anti-coagulation factors because AT-III inhibits the activity of several proteases in the coagulation cascade, and protein C inactivates coagulation factors such as

FVII.[3] We found that AA or MA treatments at 0.2% retained AT-III and protein C activities, which in turn benefited fibrinolysis. Apparently, the anti-coagulatory effects of these compounds could be partially ascribed to they enhance thrombolysis. These findings indicated that AA or MA could reduce the risk of diabetes associated atherogenesis and thrombosis via regulating both coagulatory and anti-coagulatory factors.

It is known that hyperglycemia raises oxidative stress, which subsequently enhances the release of vWF and fibrinogen.[25] Furthermore, ROS could stimulate platelet hyperactivity and facilitate coagulation.[4,26] Our data revealed that AA or MA could exhibit anti-oxidative activities, via suppressing protein expression of $p47^{phox}$ and $gp91^{phox}$, subunits of NADPH oxidase, to decrease ROS generation and spare GSH content in heart of diabetic mice. These results supported that AA and MA through their anti-oxidative actions diminished cardiac oxidative injury, which consequently mitigated oxidative stress in circulation and reversed hemostatic imbalance.

It is reported that the accumulation of HbA1c, CML and pentosidine in circulation and heart impairs cardiac functions.[9,27] The enhanced activity and expression of aldose reductase promoted AGEs formation and caused heart failure under diabetic condition.[28,29] In this present study, AA or MA intake markedly decreased protein expression of this enzyme in heart of diabetic mice, which subsequently reduced fructose flux and AGEs production. These findings suggest that these triterpenoids could abate cardiac glycative injury by inhibiting aldose reductase. On the other hand, GLO-1 is responsible for the detoxification of methylglyoxal to D-lactate.[10,30] Our data revealed that AA or MA could reserve cardiac expression of GLO-1, decrease methylglyoxal level and increase D-lactate formation. Thus, the observed lower cardiac CML and pentosidine levels in AA or MA-treated diabetic mice

could be partially ascribed to these compounds enhance glyoxalase pathway. These findings suggest that these two triterpenoids are potent GLO-1 enhancers.

The activation of NF-κB and MAPK pathways by ROS and/or AGE-RAGE interaction exacerbates the progression of inflammation, endothelial dysfunctions and hypercoagulation in heart and/or other organs through accelerating the formation of glycative, oxidant, inflammatory and even coagulant factors.[31-33] It is reported that the increased release of inflammatory cytokines and chemoattractants promoted the occurrence of diabetic heart failure.[34] Our data revealed that AA or MA treatments already decreased cardiac production of ROS, AGEs and RAGE, which in turn diminished the interaction of AGEs and RAGE, and led to less activation of NF-κB and MAPK molecules. Consequently, it was reasonable to observe the limited expression of NF-κB p50, p65, p-p38 and p-ERK1/2 in heart of diabetic mice with AA or MA treatments. Meanwhile, the decreased release of IL-6, TNF-alpha and MCP-1 in heart of those diabetic mice also agreed that NF-κB and MAPK pathways have been suppressed. Since cardiac oxidative, inflammatory and glycative injury in diabetic mice has been ameliorated, the improvement in cardiac functions, evidenced by the changes in CPK and LDH activities, could be explained. On the other hand, it is possible that these agents decreased the interaction among glycative, inflammatory and oxidative factors in circulation and heart, which subsequently alleviated cardiac damage. The other possibility is that the intake of these triterpenoids also led to their presence in other organs, and protected those tissues to reduce systemic oxidative, inflammatory and glycative responses, which contributed to mitigate cardiac injury. The improvement in glycemic control, body weight, feed and water intake in these AA or MA treated diabetic mice seemingly agreed that the overall oxidative, glycative and inflammatory stresses have been attenuated.

AA and MA are triterpenoids naturally occurring in many plant foods. The intake of these agents at 0.2% for mice is approximately equal to 28 g/day for a 70-kg adult. Our previous study[14] and present study indicated that dietary intake of these triterpenoids increased their bioavailability in organs including heart and kidney; and did not cause any toxic sign. Thus, these triterpenoids might be safe and feasible for application. However, we found that diabetic mice had lower AA or MA deposit in hearts when compared with normal mice. Both AA and MA are lipophilic compounds; thus, they might enhance metabolic burden under diabetic condition, and lead to lower cardiac bioavailability. Thus, the supplement of these agents for diabetic subjects should consider their metabolic capability.

In summary, dietary intake of asiatic acid or maslinic acid at 0.2% markedly declined NADPH oxidase, polyol, RAGE, NF-κB and MAPK pathways in heart of diabetic mice, which in turn decreased cardiac levels of ROS, AGEs and inflammatory cytokines. Furthermore, these compounds mitigated hemostatic disorders via lowering vWF, FVII and fibrinogen levels or activity, as well as retaining protein C and AT-III activities in circulation. These findings support that the supplement of these triterpenoids or foods rich in these compounds may protect heart under diabetic condition to attenuate glycative injury and coagulatory disorders.

Conflict of interest statement

There was no Conflict of Interest regarding this manuscript.

Acknowledgement

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Table 1

Body weight (BW, g/mouse), water intake (WI, ml/mouse/day), feed intake (FI, g/mouse/day), heart weight (HW, mg/mouse) and cardiac content (μg/g) of AA or MA in normal or diabetic mice treated with AA or MA at 0, 0.1 or 0.2% for 12 weeks. Values are mean ± SD, n=10.

	Normal			DM				
		AA, 0.2	MA, 0.2		AA, 0.1	AA, 0.2	MA, 0.1	MA, 0.2
BW	30.6±2.0^{d}	29.7±1.5^{d}	30.2±2.2^{d}	12.8±0.9^{a}	14.9±0.5^{b}	18.6±1.0^{c}	15.3±0.7^{b}	19.2±1.3^{c}
WI	2.3±0.5^{a}	2.5±0.6^{a}	2.2±0.4^{a}	6.1±1.0^{c}	5.4±0.8^{c}	4.0±0.4^{b}	5.5±0.8^{c}	3.7±0.7^{b}
FI	2.2±0.7^{a}	2.0±0.8^{a}	2.3±0.5^{a}	5.8±1.2^{d}	4.6±0.6^{c}	3.4±0.5^{b}	4.3±0.7^{c}	3.2±0.8^{b}
HW	149±7^{a}	151±5^{a}	147±8^{a}	152±4^{a}	146±9^{a}	150±6^{a}	148±10^{a}	145±5^{a}
AA	-*,^{a}	1.34±0.11^{c}	-^{a}	-^{a}	-^{a}	0.88±0.08^{b}	-^{a}	-^{a}
MA	-^{a}	-^{a}	1.29±0.07^{c}	-^{a}	-^{a}	-^{a}	-a	0.76±0.05^{b}

*Means too low to be detected.

$^{a\text{-}d}$Means in a row without a common letter differ, $p<0.05$.

Table 2

Plasma level of glucose (mmol/l), HbA1c (%), insulin (nmol/l), CPK (IU/l), LDH (IU/l), ALT (U/l) and AST (U/l) in normal or diabetic mice treated with AA or MA at 0, 0.1 or 0.2% for 12 weeks. Values are mean ± SD, n=10.

	Normal			DM				
		AA, 0.2	MA, 0.2		AA, 0.1	AA, 0.2	MA, 0.1	MA, 0.2
Glucose	9.3±0.3^{a}	9.1±0.5^{a}	9.4±0.7^{a}	28.2±2.3^{d}	23.0±1.5^{c}	17.1±0.9^{b}	21.7±1.1^{c}	16.0±0.8^{b}
HbA1c	2.5±0.4^{a}	2.7±0.5^{a}	2.4±0.3^{a}	12.1±1.2^{d}	9.2±0.9^{c}	6.5±0.7^{b}	9.6±1.0^{c}	7.0±0.6^{b}
Insulin	14.1±0.7^{c}	14.7±0.6^{c}	14.3±0.5^{c}	5.3±0.4^{a}	6.2±0.7^{a}	7.4±0.8^{b}	6.0±0.6^{a}	7.7±0.3^{b}
CPK	56.7±2.0^{a}	54.2±1.7^{a}	57.0±2.3^{a}	202.2±13.8^{d}	164.7±10.8^{c}	113.5±9.6^{b}	156.3±5.3^{c}	109.6±7.1^{b}
LDH	32.5±1.8^{a}	33.4±1.2^{a}	34.1±1.5^{a}	166.4±11.0^{d}	129.5±8.5^{c}	90.3±6.7^{b}	132.8±10.4^{c}	96.8±7.2^{b}
ALT	26±3^{a}	28±5^{a}	27±4^{a}	30±3^{a}	25±4^{a}	27±3^{a}	29±3^{a}	28±2^{a}
AST	33±4^{a}	35±2^{a}	35±3^{a}	36±2^{a}	34±2^{a}	35±4^{a}	33±2^{a}	34±3^{a}

$^{a-d}$Means in a row without a common letter differ, $p<0.05$.

Table 3

Plasma level of vWF (%) and fibrinogen (g/l); and activity of FVII activity (%), PAI-1 (kU/l), AT-III (%) and protein C (%) in normal or diabetic mice treated with AA or MA at 0, 0.1 or 0.2% for 12 weeks. Values are mean ± SD, n=10.

	Normal			DM				
		AA, 0.2	MA, 0.2		AA, 0.1	AA, 0.2	MA, 0.1	MA, 0.2
vWF	100^{a}	98 ± 4^{a}	97 ± 3^{a}	411 ± 23^{f}	352 ± 16^{e}	267 ± 9^{c}	318 ± 10^{d}	198 ± 12^{b}
Fibrinogen	2.38 ± 0.11^{a}	2.25 ± 0.13^{a}	2.23 ± 0.10^{a}	5.08 ± 0.26^{c}	4.95 ± 0.19^{c}	3.91 ± 0.15^{b}	5.02 ± 0.21^{c}	4.04 ± 0.13^{b}
FVII	100^{a}	96 ± 3^{a}	99 ± 2^{a}	313 ± 19^{c}	304 ± 12^{c}	225 ± 15^{b}	301 ± 7^{c}	208 ± 10^{b}
PAI-1	7.1 ± 0.6^{a}	7.3 ± 0.5^{a}	6.9 ± 0.7^{a}	20.2 ± 1.3^{b}	19.5 ± 1.0^{b}	19.0 ± 0.8^{b}	19.2 ± 0.7^{b}	19.1 ± 1.2^{b}
AT-III	142 ± 10^{d}	139 ± 7^{d}	144 ± 8^{d}	68 ± 2^{a}	83 ± 5^{b}	105 ± 9^{c}	80 ± 4^{b}	107 ± 7^{c}
Protein C	98 ± 5^{c}	95 ± 8^{c}	97 ± 6^{c}	54 ± 4^{a}	60 ± 5^{a}	79 ± 3^{b}	59 ± 2^{a}	77 ± 4^{b}

[a-f]Means in a row without a common letter differ, $p<0.05$.

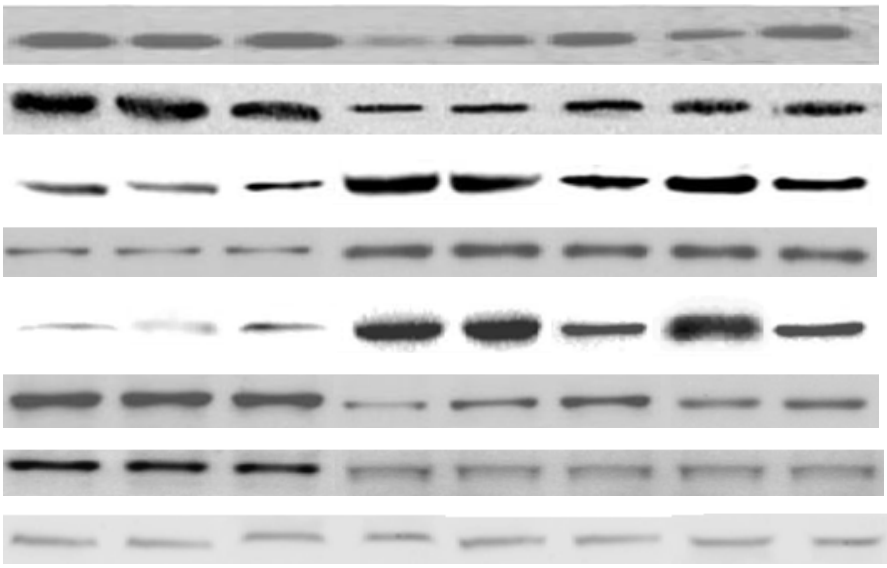
Table 4

Cardiac level of GSH (nmol/mg protein), ROS (RFU/mg protein), CML (pmol/mg protein), pentosidine (pmol/mg protein), fructose (nmol/mg protein), methylglyoxal (nmol/mg protein) and D-lactate (nmol/mg protein) in normal or diabetic mice treated with AA or MA at 0, 0.1 or 0.2% for 12 weeks. Values are mean ± SD, n=10.

	Normal			DM				
		AA, 0.2	MA, 0.2		AA, 0.1	AA, 0.2	MA, 0.1	MA, 0.2
GSH	20.3±1.1^{d}	21.0±1.3^{d}	20.6±0.8^{d}	8.8±0.7^{a}	11.5±0.9^{b}	15.2±1.2^{c}	12.0±0.6^{b}	14.7±1.0^{c}
ROS	0.24±0.08^{a}	0.27±0.05^{a}	0.25±0.04^{a}	1.25±0.14^{d}	0.90±0.11^{c}	0.63±0.07^{b}	0.95±0.12^{c}	0.59±0.06^{b}
CML	8.2±0.8^{a}	8.0±1.0^{a}	7.8±1.3^{a}	79.4±4.2^{f}	66.8±2.6^{e}	53.2±3.1^{c}	60.3±2.9^{d}	45.1±3.3^{b}
Pentosidine	0.21±0.04^{a}	0.19±0.5^{a}	0.22±0.6^{a}	1.42±0.16^{d}	1.15±0.11^{c}	0.78±0.09^{b}	1.07±0.13^{c}	0.80±0.10^{b}
Fructose	17.1±1.4^{a}	16.8±1.2^{a}	17.3±1.5^{a}	90.1±4.7^{d}	72.3±3.5^{c}	46.2±2.1^{b}	69.5±2.9^{c}	43.0±3.1^{b}
Methylglyoxal	0.68±0.04^{a}	0.65±0.05^{a}	0.63±0.03^{a}	6.63±0.28^{d}	4.51±0.16^{c}	2.06±0.13^{b}	4.39±0.09^{c}	2.17±0.21^{b}
D-lactate	79.2±2.5^{d}	80.5±1.9^{d}	81.1±2.7^{d}	49.5±1.3^{a}	57.3±1.7^{b}	66.7±2.0^{c}	59.2±1.5^{b}	68.1±1.8^{c}

$^{a\text{-}f}$Means in a row without a common letter differ, $p<0.05$.

Fig. 1. Cardiac expression of $p47^{phox}$, $gp91^{phox}$, AR, SDH, RAGE, GLO-1 and GLO-2 in normal or diabetic mice treated with AA or MA at 0, 0.1 or 0.2% for 12 weeks was detected by western blot analyses. GAPDH was used as loading control. $p47^{phox}$, $gp91^{phox}$, AR, SDH, RAGE, GLO-1 and GLO-2 protien bands were normalized to GAPDH expression. A representative image is shown. Each bar represents the mean ± SD, n=10. $^{a-d}$Means among bars without a common letter differ, $p<0.05$.



DM	-	-	-	+	+	+	+	+
AA	-	0.2	-	-	0.1	0.2	-	-
MA	-	-	0.2	-	-	-	0.1	0.2

1

2

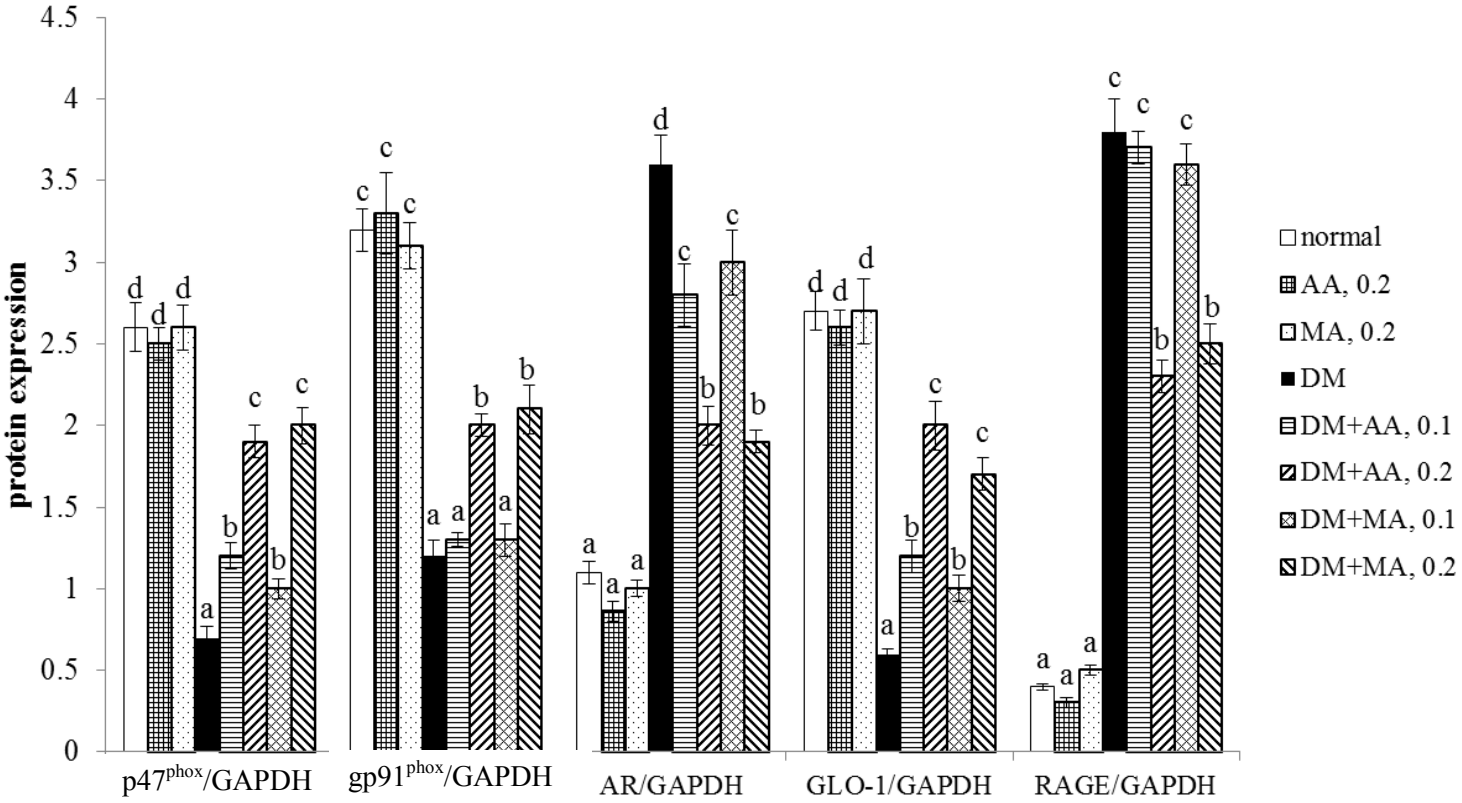


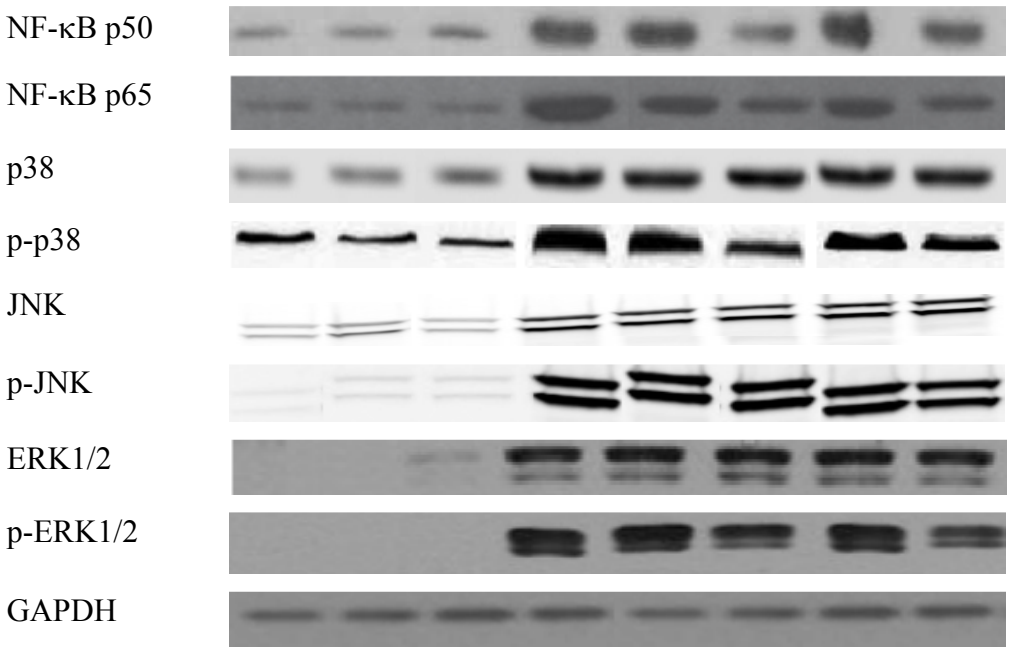
Table 5

Cardiac level (pg/ml) of MCP-1, IL-6 and TNF-alpha in normal or diabetic mice treated with AA or MA at 0, 0.1 or 0.2% for 12 weeks. Values are mean ± SD, n=10.

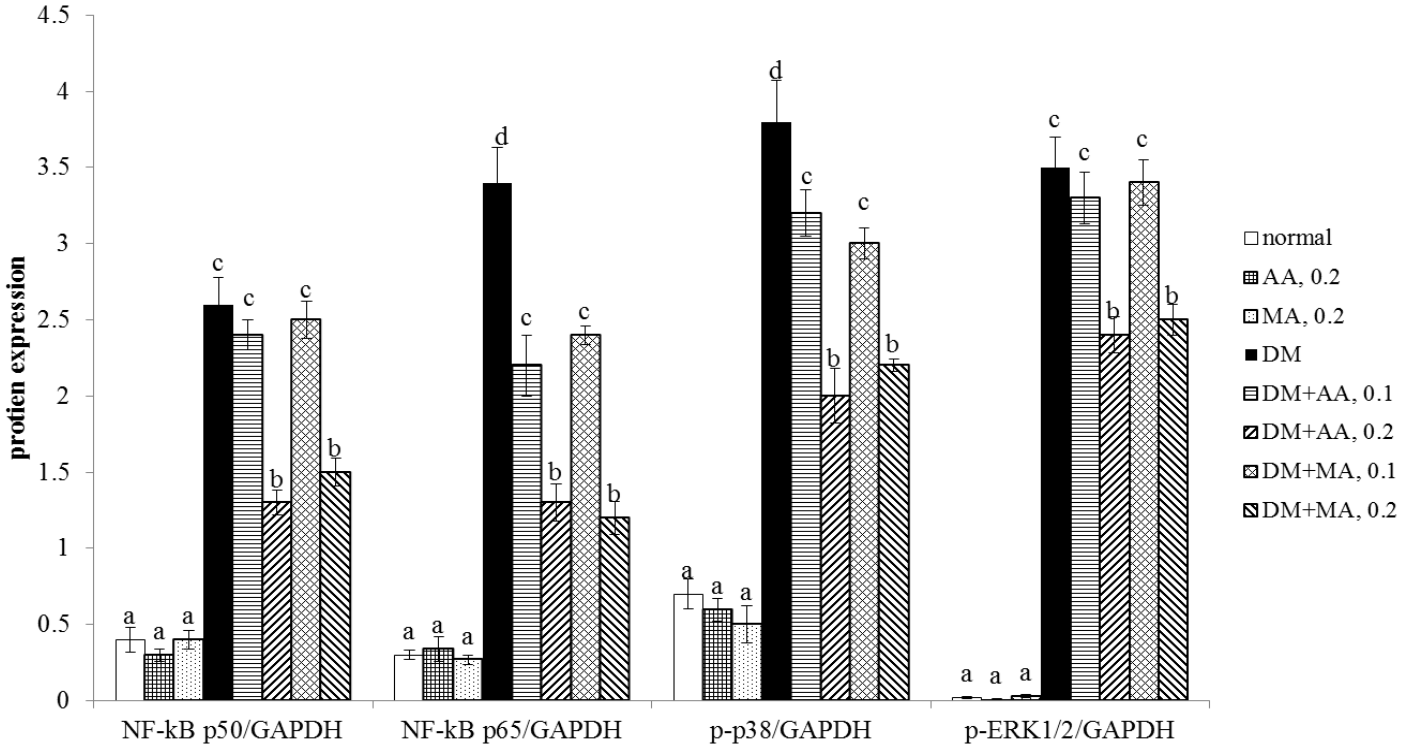
	Normal			DM				
		AA, 0.2	MA, 0.2		AA, 0.1	AA, 0.2	MA, 0.1	MA, 0.2
MCP-1	12.2±1.3[a]	10.5±1.2[a]	11.4±1.1[a]	79.5±4.2[d]	58.1±2.6[c]	41.6±2.9[b]	55.9±3.0[c]	38.3±2.5[b]
IL-6	13.4±0.8[a]	12.8±1.5[a]	11.9±1.2[a]	92.1±3.9[d]	74.7±3.0[c]	47.8±3.1[b]	75.2±1.8[c]	45.2±2.0[b]
TNF-alpha	11.6±1.0[a]	11.0±1.4[a]	10.7±0.7[a]	108.6±5.0[d]	80.2±3.6[c]	52.6±3.3[b]	76.1±2.2[c]	48.6±2.7[b]

[a-d]Means in a row without a common letter differ, $p<0.05$.

Fig. 2. Cardiac expression of NF-κB and MAPK in normal or diabetic mice treated with AA or MA at 0, 0.1 or 0.2% for 12 weeks was detected by western blot analyses. GAPDH was used as loading control. NF-κB and MAPK protien bands were normalized to GAPDH expression. A representative image from each group is shown. Each bar represents the mean ± SD, n=10. [a-d]Means among bars without a common letter differ, $p<0.05$.



DM	-	-	-	+	+	+	+	+
AA	-	0.2	-	-	0.1	0.2	-	-
MA	-	-	0.2	-	-	-	0.1	0.2



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