The effect of advanced glycation end products (AGE) breaker, alagebrium on the cardiac structural and functional change in type I diabetic rat model

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Directed by Professor Namsik Chung

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I dedicate the dissertation to my loving family.

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<ABSTRACT>

The effect of advanced glycation end products (AGE) breaker, alagebrium on the cardiac structural and functional change in type I diabetic rat model

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Chronic hyperglycemia contributes to diabetic complications through the formation of advanced glycation end products (AGE). The crosslink breaker, 3-phenacyl-4,5-dimethylthiazolium chloride (ALT-711), alagebrium chloride is known to reduce the pathological condition through breaking AGE cross-links. However, there has been little assessment regarding the impact of alagebrium on AGE-dependent signaling or restoring structural molecular physiology. There are also few studies about the functional change to couple with the structural and molecular investigation. We investigated the effect of alagebrium on myocardial functional change using echocardiography as well as structural change in diabetic rat model based on the cellular mechanism**.** AGE enhanced reactive oxygen species (ROS) synthesis dose-dependently in cardiomyocytes. Alagebrium inhibited ROS synthesis enhancement by AGE and increased expression of receptor for AGE (RAGE). In western blot and

RT-PCR experiments, alagebrium also inhibited AGE-induced activation of MAPKinase and decreased AGE-induced connective tissue growth factor (CTGF) and extracellular matrix. In echocardiography, the left ventricular (LV) end-diastolic dimension was increased (6.33 ±0.58mm at initial vs. 8.33±0.58mm at 16weeks after diabetes, p=0.003), and systolic strain in circumferential and radial direction (47.7±5.5% at initial vs. 15.2±5.8% at 16weeks after diabetes, p=0.048) was decreased as diabetes was progressed. Diabetic progression also modified collagen structure. However, the alagebrium treatment during 4weeks reversed these LV remodeling (8.33±0.58mm before alagebrium vs. 7.33±0.58mm after alagebrium, p=0.099) and improved myocardial systolic function, that is, radial strain was increased from 15.2±5.8% before alagebrium to 38.7±6.9% after alagebrium treatment with borderline statistical significance (p=0.05). In conclusion, alagebrium treatment in diabetic rat heart improved myocardial systolic dysfunction and LV remodeling by blocking the AGE-induced intracellular ROS synthesis. The beneficial effect of alagebrium on diabetic rat heart was mediated by reduction of extracellular matrix production and this might be mediated by decreased activation of ERK (extracellular signal-regulated kinase) MAP kinase.

Key words: advanced glycation end products, crosslink breaker (ALT-711), diabetic myocardium

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I. Introduction

Diabetes Mellitus (DM) is an established risk factor for cardiovascular events such as coronary heart disease, left ventricular hypertrophy (LVH), cardiac fibrosis and these abnormalities contribute to the development of diastolic and systolic dysfunction and ultimately heart failure.[1]

Chronic hyperglycemia contributes to diabetic complications through the formation of advanced glycation end products(AGEs) which are irreversibly formed biochemical end products of nonenzymatic glycation.[2] These AGEs include n-carboxymethyllysine (CML), known to be involved in diabetic nephropathy; pentosidine, which is seen in patients with diabetes and chronic inflammatory disorders; and the imidazolium salt cross-link, glyoxal-lysine dimer (GOLD), present in the serum of diabetic patients.[3] It has been established that AGE form irreversible cross-links with protein backbones

such as collagen and elastin as they accumulate in tissues.[3] And this is a pathophysiological event that has been recognized as a causative factor in diabetic complications and age-related diseases. [4-6]

AGE and protein cross-linking activate growth factors such as Tissue Growth Factor-β1 (TGF-β1) and Connective Tissue Growth Factor (CTGF), induce a number of processes, and initiate inflammatory reactions. Many cellular responses elicited by AGE have been reported to have deleterious effects on organ structure and function.[7] In particular, AGE act as ligands to various AGE receptors (RAGE, AGE-R1, AGE-R2, AGE-R3) that are present on macrophages, epithelial cells, mesangial cells, and endothelial cells. This interaction triggers a number of effects, including an inflammatory response, angiogenesis, increased expression of prosclerotic growth factors (TGF-β and CTGF), and induction of extracellular matrix protein production.[8-10] Candido et al[11] found that expression of RAGE and AGE-R3 was upregulated in diabetic rat hearts.

Interventions to reduce AGE-mediated injury aim to chemically break existing AGE-formed cross-links as well as to prevent the formation of new cross-links. The crosslink breaker, 3-phenacyl-4,5-dimethylthiazolium chloride (ALT-711), alagebrium chloride breaks AGE cross-links without disrupting the natural glycation sited or peptide bonds. It improves arterial stiffness and ventricular function in preclinical trials[12,13] and vascular compliance in humans[14]. In experimental diabetes, alagebrium reversed large artery stiffness[15] and restored LV ejection fraction. LV collagen solubility and cardiac BNP in association with reduced cardiac AGE levels was improved and the increase in RAGE, AGE-R3, CTGF, and collagen III expression was abrogated.[11,16] All these datas suggest a potential role for crosslink breakers in the prevention and treatment of diabetic cardiac complication.

However, previous studies have some limitations. There has been little

assessment regarding the impact of alagebrium on AGE-dependent signaling or restoring structural molecular physiology. There hasn't been also analyzed about the functional change to couple with the structural and molecular investigation.

Therefore, we investigated the effect of alagebrium on myocardial functional change using echocardiography[17-19] as well as structural change in diabetic rat model based on the cellular mechanism in this study. Effect of AGE on the formation of intracellular reactive oxygen species (ROS) in cardiomyocytes and the effect of alagebrium on the formation of intracellular ROS was evaluated. The involvement of alagebrium and MAPK kinase in AGE-stimulated cardiomyocytes was examined. Moreover, inhibitory effects of alagebrium on expression of CTGF and extracellular matrix in AGE-stimulated cardiomyocytes were also analyzed.

II. Materials and methods

1. Animal experiments

Forty eight-week old male, Sprague-Dawley rats weighing 200 to 250 grams received Streptozotocin (SZ) at 50 mg/kg intraperitoneally. The diabetic rats whose sugars were more than 200 mg/dL 2 days after SZ injection received long acting insulin 2 U/day daily. We performed RT-PCR for collagen I, III, conncective tissue growth factor (CTGF) and made echocardiography every 4 week until 16 weeks after DM induction to evaluate the sequential change of diabetic myocardium. Sixteen weeks later, the rats were randomized into two study groups: Ten diabetic Sprague-Dawley rats receiving placebo and 10 diabetic Sprague-Dawley rats receiving algebrium 10 mg/kg. The Algebrium chloride was mixed with pulverized standard chow and treated for 4 weeks. After 4 weeks, we made various analyses to know the effects of alabebrium on diabetic myocardium.

2. Isolation of neonatal rat cardiomyocytes

Neonatal rat cardiomyocytes were isolated and purified by previously described methods.[16] Briefly, hearts of 1-2 day-old Sprague Dawley rat pups was dissected, and the ventricles washed with Dulbecco's phosphate-buffered saline solution (pH 7.4, Gibco BRL, Grand Island, NY, USA) lacking Ca^{2+} and Mg^{2+}. Using micro-dissecting scissors, hearts was minced until the pieces are approximately 1 mm^3 and treated with 10ml of collagenase I (0.8 mg/ml, 262 units/mg, Gibco BRL, Grand Island, NY, USA) for 15 minutes at 37°C. The supernatant was then removed and the tissue was treated with fresh collagenase I solution for an additional 15 minutes. The cells in the supernatant were transferred to a tube containing cell culture medium (α-MEM containing 10% fetal bovine serum, Gibco BRL, Grand Island, NY,

USA). The tubes were centrifuged at 1200 rpm for 4 minutes at room temperature, and the cell pellet was resuspended in 5 ml of cell culture medium. The above procedures were repeated 7-9 times until there little tissue was left. Cell suspensions were collected and incubated in 100mm tissue culture dishes for 1-3 hours to reduce fibroblast contamination. The non-adherent cells were collected and seeded to achieve a final concentration of 5 x 10^5 cells/ml. After incubation for 4-6 hours, the cells were rinsed twice with cell culture medium and 0.1 µM BrdU added. Cells were then cultured with 10% (v/v) FBS in a CO_2 incubator at 37°C.[19]

3. Measurement of intracellular ROS generation

Cardiomyocytes are labeled with 2′, 7′-dichlorodihydrofluorescein diacetate (H_2DCFDA; Molecular Probe, CA, USA).[15] The probe H_2DCFDA (5M) enters the cell and the acetate group on H_2DCFDA is cleaved by cellular esterases, trapping the non-fluorescent 2′, 7′-dichlorofluorescin (DCFH) inside. Subsequent oxidation by reactive oxygen species yields the fluorescent product DCF. The dye, when exposed to an excitation wavelength of 480 nm, emits light at 535 nm only when it has been oxidized. Labeled rat aortic cardiac muscle cells are examined using a luminescence spectrophotometer for oxidized dye. The quiescent cells were treated with alagebrium or/and AGE before labeling with H_2DCFDA.[21]

4. RT-PCR analysis

The expression levels of various proteins were analyzed by reverse transcription polymerase chain reaction (RT-PCR). Total RNA was prepared by the UltraspectTM-II RNA system (Biotecx Laboratories, Inc., TX, USA) and single-stranded cDNA was then synthesized from isolated total RNA by Avian Myeloblastosis virus (AMV) reverse transcriptase. A 20 µl reverse

transcription reaction mixture containing 1 g of total RNA, 1X reverse transcription buffer (10 mM Tris-HCl, pH 9.0, 50 mM KCl, 0.1% Triton X-100), 1 mM deoxynucleoside triphosphates (dNTPs) 0.5 unit of RNase inhibitor, 0.5 g of oligo(dT)15 and15 units of AMV reverse transcriptase was incubated at 42℃ for 15 min, heated to 99℃ for 5 min, and then incubated at 0-5℃ for 5 min. PCRs were performed for 35 cycles with 3' and 5' primers based on the sequences of various genes. GAPDH was used as the internal standard. The signal intensity of the amplification product was normalized to its respective GAPDH signal intensity.[22,23]

Primers were as follows.

Table 1. Used primer list

primer	sequence	size (bp)
RAGE	5'-GGCCTTCCTCGGCGCAGACC-3'	260
	5'-TAGATGCCCTCATCCTCATGC-3'	
Collagen type III	5'-AGATGCTGGTGCTGAGAAG-3'	312
	5'-TGGAAAGAAGTCTGAGGAAGG-3'	
Fibronectin	5'-GTGAA-GAACG-AGGAG-GATGT-G-3'	300
	5'-GTGATGGCGGATGATGTAGC-3'	
Connective tissue	5'-AAGAAGACTCAGCCAGACC-3'	235
growth factor	5'-AGAGGAGGAGCACCAAGG-3'	
GAPDH	5'-ACCACAGTCCATGCCATCAC-3'	450
	5'-TCCACCACCCTGTTGCTGTA-3'	

5. Immunoblot analysis

Cardiomyocytes were treated with Alagebrium before the addition of advanced glycation endproduct as stimulus. Cells were washed once in PBS and lysed in a lysis buffer (Cell signaling, Beverly, MA, USA) containing 20 mM Tris (pH 7.5), 150 mM NaCl, 1 mM Na_2EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na_3VO_4, 1

mg/ml leupeptin and 1 mM PMSF. Protein concentrations were determined using the Bradford protein assay kit (BioRad, Hercules, CA, USA). Proteins were separated in a 12% SDS-polyacrylamide gel and transferred to PVDF membrane (Millipore Co, Bedford, MA, USA). After blocking the membrane with Tris-buffered saline-Tween 20 (TBS-T, 0.1% Tween 20) containing 5% non-fat dried milk for 1 hr at room temperature, membranes were washed twice with TBS-T and incubated with primary antibodies for 1 hr at room temperature or for overnight at 4°C. The following primary antibodies were used: rabbit anti-Extracellular signal-Regulated Kinase (ERK), mouse anti-phospho ERK (Santa Cruz Biotechnology, Santa Cruz, CA, USA). The membrane were washed three times with TBS-T for 10 min, and then incubated for 1 hr at room temperature with horseradish peroxidase (HRP)-conjugated secondary antibodies. After extensive washing, the bands were detected by enhanced chemiluminescence (ECL) reagent (Santa Cruz Biotechnology, Santa Cruz, CA, USA). The band intensities were quantified using a Photo-Image System (Molecular Dynamics, Uppsala, Sweden).[24,25]

6. Immunohistochemistry

The heart was perfusion-fixed with 10% (v/v) neutral buffered formaldehyde for 24h, transversely sectioned into four comparably thick sections, and embedded in paraffin by routine methods. Sections of 2 μm-in thicknesses were mounted on a gelatin-coated glass slides to ensure different stains. After deparaffinization and rehydration, the sections were stained with hematoxylin and eosin to assess cytologic details, such as nuclei, cytoplasm and connective tissue. Other serial sections were analyzed with mouse anti-type I collagen, rabbit anti-type III collagen (Upstate Biotechnology, NY, USA), and rabbit anti-RAGE (Santa Cruz Biotechnology Inc., CA, USA). Fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG (Jackson

ImmunoResearch Lab., PA, USA) and Texas red-conjugated goat anti-mouse IgG or mouse anti-goat IgG (Jackson ImmunoResearch Lab., PA, USA) were used as secondary antibody. All images were made by using an excitation filter under reflected light fluorescence microscopy and transferred to a computer equipped with MetaMorph software ver. 4.6 (Universal Imaging Corp.).[25]

7. Echocardiographic analysis

A. Two-dimensional and Doppler Echocardiography

Echocardiography was performed using the ultrasound system (Vivid 7 GE Vingmed, Horten, Norway) with a 2.5-MHz transducer. Standard 2-dimensional parameters (end-diastolic, end-systolic dimensions, ventricular septum, posterior wall thickness, LV mass index and ejection fraction[27]) were measured. In our experiments, we determined that reliable and consistent transthoracic images were available only from the parasternal short-axis view. This view allows visualization of the anterior, septum, inferior, and inferolateral segments at the mid-ventricular level.

B. Strain and strain rate imaging acquisition

Strain echocardiography measures the extent (strain) and the rate (strain rate) of regional myocardial shortening and lengthening.[28] The analysis was performed using the EchoPac PC software for offline analysis of speckle tracking imaging.[28] Two-dimensional strain is a novel non-Doppler-based method to evaluate systolic strain from standard bidimensional acquisitions. A sample region (strain distance 5 mm) was placed in the endocardial portion of each segment to yield peak strains in radial and circumferential direction. Average circumferential and radial strains were calculated for the six mid-LV segments.

8. Statistical analysis

Data are presented as mean ± S.E.M. of more than three separate experiments performed in triplicate. Where results of blots and RT-PCR are shown, a representative experiment is depicted. Comparisons between multiple groups were performed with one-way ANOVA (Analysis of Variance) with Bonferroni's test. Statistical significance was defined as $p < 0.05$ and $p < 0.01$.

III. RESULTS

1. Effect of AGE on the formation of intracellular ROS in cardiomyocytes

To investigate the effect of AGE on the formation of intracellular reactive oxygen species (ROS), cells were treated with various concentrations (0-1000 μg/ml) of AGE at high (25.5 mM) or low (5.5 mM) extracellular glucose condition. Following 3 hours incubation of AGE, the ROS production of neonatal rat cardiomyocytes was increased dose dependently and high extracelluar glucose condition significantly enhance ROS generation, indicating that AGE and glucose level could be one of stimulators in ROS production (Figure 1A). To further confirm whether incubation time of AGE might regulate the ROS production of cardiomyocytes, cells were treated with 50 μg AGE for various times. Figure 1B shows that the ROS formation was generated in a time-dependent manner.

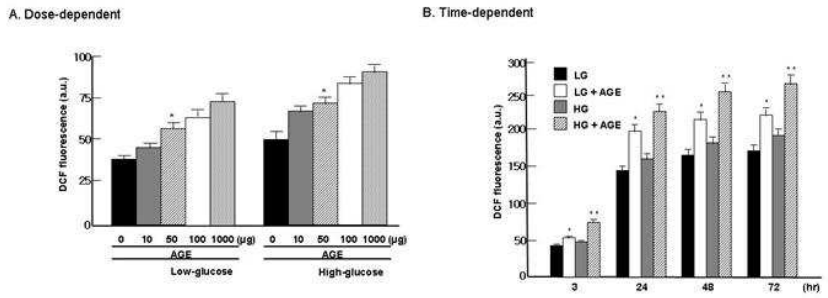

Figure 1. Effect of advanced glycation end products (AGEs) on the formation of intracellular reactive oxygen species (ROS) in cardiomyocytes (AGE: advanced glycosylation end products; LG: low glucose (5.5 mM), HG: high glucose (25.5 mM) . * $p < 0.05$ vs Control

2. Effect of alagebrium on the formation of intracellular ROS in cardiomyocytes treated with AGE

In order to investigate whether alagebrium might modify the effect of AGE on the ROS production of cardiomyocytes, cells were pretreated with various concentrations of alagebrium for 2 hrs prior to exposure of AGE (50 μg/ml) at high (25.5 mM) or low (5.5 mM) extracellular glucose condition. As shown in Figure 2, AGE and glucose level as stimulators enhanced ROS formation in cardiomyocytes but alagebrium was reduced the AGE-induced increase in reactive oxygen species in a dose-dependent manner at both extracellular glucose conditions. In high extracellular glucose condition, the production of ROS was sharply reduced by treatment of alagebrium compared with low extracellular glucose condition.

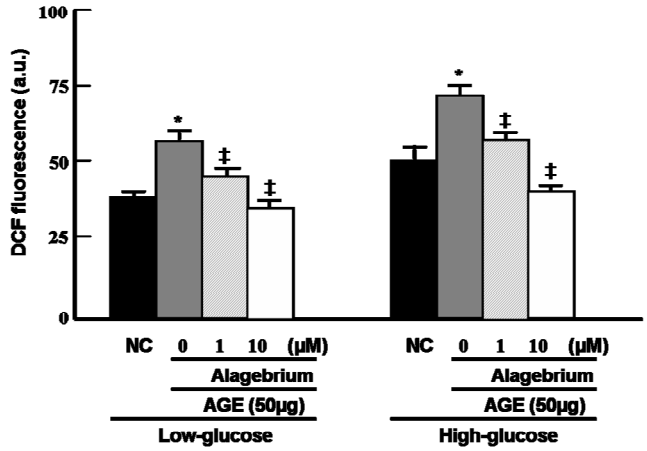

Figure 2. Effect of alagebrium on the formation of intracellular reactive oxygen species (ROS) in cardiomyoctes treated with advanced glycation end products (AGEs). * $p < 0.05$ vs Control, ‡ < 0.05 vs group with only AGE

3. Effect of alagebrium on expression of RAGE in cardiomyocytes stimulated by AGE

In diabetes, it is postulated that the increase in AGEs activates the expression of AGE receptors. No such data on RAGE distribution of cardiomyocytes in the diabetic condition has been previously reported. To determine whether alagebrium regulated the expression of receptor for AGE (RAGE), cells were pretreated with with 1 and 10 μM of alagebrium for 2 hrs prior to exposure of AGE. AGE induced the sharply increased expression of RAGE in cardiomyocyte but alagebrium significantly reduced RAGE gene expression compared with untreated control and the treatment with 10 μM alagebrium had a significant decrease in gene expression for RAGE in cells compared with 1 μM alagebrium. (Figure 3)

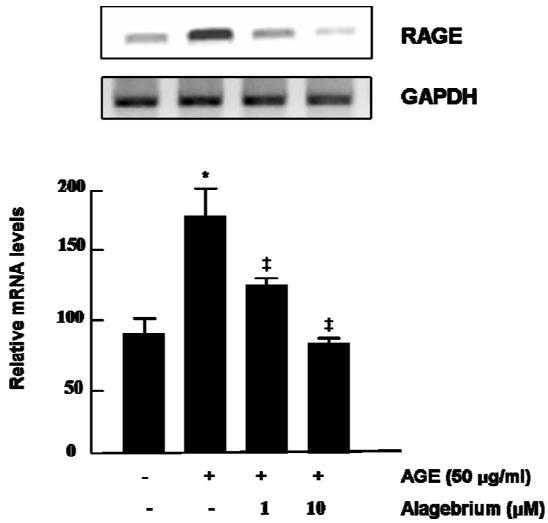

Figure 3. Effect of alagebrium on the expression of RAGE in cardiomyocytes treated with advanced glycation end products (AGEs) (RAGE: receptor for advanced glycation end products AGE: advanced glycation end products). * p <0.05 vs Control, ‡ <0.05 vs group with only AGE

4. Effect of alagebrium on activation of ERK in cardiomyocytes stimulated by AGE

It has been known that AGE stimulates the activation of ERK MAPK in cardiomyocytes. In order to investigate the effect of alagebrium on ERK MAPK activation in AGE-stimulated cardiomyocytes, the change of ERKs phosphorylation (42 and 44 kDa) was detected by immunoblot assay. We demonstrated that ERK MAPK activation was seen within 30 min with 50 μg/ml of AGE. These signaling activations were significantly inhibited by pretreatment with 1 and 10 μM of alagebrium and the activation of ERKs was dose-dependently reduced. (Figure 4)

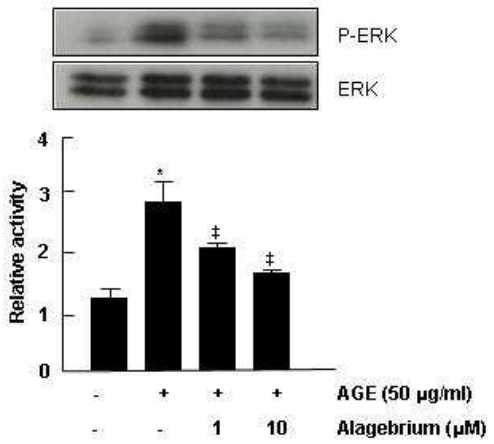

Figure 4. Effect of alagebrium on the phosphorylation of MAP kinase in cardiomyocytes treated with advanced glycation end products (AGEs) (ERK: extracellular signal-regulated kinase; AGE: advanced glycation end products). * $p < 0.05$ vs Control, ‡ < 0.05 vs group with only AGE

5. Inhibitory effects of alagebrium on expression of CTGF and extracellular matrix in AGE-stimulated cardiomyocytes

To demonstrate whether a direct cellular mechanism was involved in the regulatory effect of alagebrium on AGE-induced connective tissue growth factor (CTGF) and extracellular matrix (ECM) expression in cardiomyocytes, we performed experiments in cultured cardiomyocytes. As illustrated in Figure 5, AGE (50 μg/ml) treatment for 24 h markedly increased the mRNA expression of CTGF in cardiomyoctes compared with untreated control. Alagebrium prevented upregulation of CTGF expression compared with untreated cells with mRNA levels similar to those observed in normal control. The treatment of AGE had a significant increase in extracellular matrix protein expression such as collagen type III and fibronectin compared with normal control. AGE had a 2-fold increase in cardiac collagen type III and fibronectin gene expression compared with normal control but alagebrium (1and 10 μM) dose dependently abolished this increase of gene expression. (Figure 5)

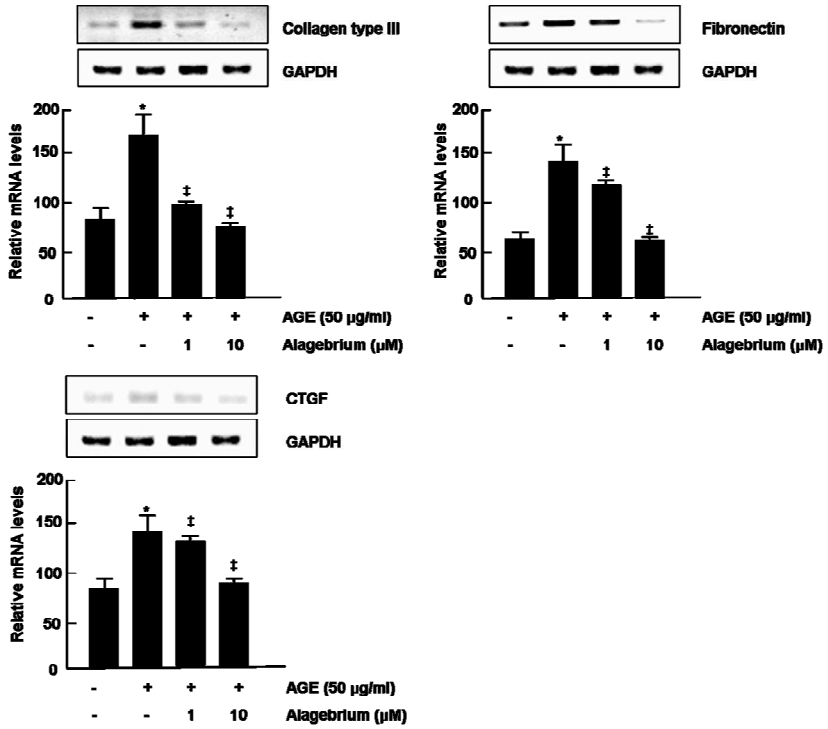

Figure 5. Inhibitory effects of alagebrium on expression of Collagen III, fibronectin and connective tissue growth factor (CTGF) in cardiomyocytes treated with advanced glycation end products (AGEs). * p <0.05 vs Control, ‡ <0.05 vs group with only AGE

6. The structural and functional change of diabetic myocardium as diabetes progression

The left ventricular dimension was gradually larger as diabetes was progressed (p=0.045) as shown in table 2. The value of normal rat and 4weeks-rat was significantly different from 16weeks rat with p <0.05. This difference was less apparent at 8 weeks with p <0.1, that is, the significant LV remodeling was prominent at DM 8weeks. Although there was no significant difference in ejection fraction between the groups, the systolic radial strain

was significantly lower at 16 weeks compared to normal and 4weeks (Figure 6). We found the systolic dysfunction in circumferential and radial direction was progressed as age and DM. (Figure 7) In that, diabetes is associated with both diastolic and systolic dysfunction. It remains to be determined if the altered cardiac performance in diabetes involves alterations in myocardial collagen structure. The expression of collagen type I and III in diabetic heart was increased with diabetic progression and the increased extent of gene expression was time-dependent. Moreover, CTGF, one of prosclerotic cytokines was also increased with diabetic progression. (Figure 8)

Table 2. Echocardiographic parameters with diabetes progression, DM: diabetes mellitus LVIDd: left ventricular end-diastolic dimension; EF: ejection fraction; C: circumferential; R: radial; SR: strain rate. † : $p<0.05$ significant difference compared to DM 16 weeks, ‡ : $p< 0.1$ significant difference compared to DM 16 weeks

	Normal (n=4)	DM 4wks (n=4)	DM 8wks (n=4)	DM 12wks (n=3)	DM 16wks (n=3)	P value
LVIDd (mm)	6.33±0.58†	6.33±0.58†	6.50±0.71‡	7.67±0.58	8.33±0.58	0.045
EF (%)	69.5±3.6	71.0±6.0	76.9±8.4	70.5±2.4	71.7±10.5	0.833
C strain (%)	-16.0±1.68†	-16.2±4.13†	-15.0±2.88‡	-12.4±1.02	-8.5±2.78	0.058
C systolic SR (1/s)	-3.10±0.56	-3.44±0.64	-3.15±0.24	-2.19±0.32†	-3.62±0.74	0.138
C diastolic SR (1/s)	3.85±0.69	2.41±0.72	3.08±1.17	2.66±0.93	2.74±0.48	0.308
R systolic strain (%)	47.7±5.50†	48.3±18.71†	39.5±11.71	23.8±10.31	15.21±5.75	0.048
R systolic SR (1/s)	6.62±0.71	7.97±1.98	8.18±0.67‡	5.16±1.03	4.82±2.29	0.077
R diastolic SR (1/s)	-5.53±1.14	-5.00±1.46	-6.51±1.87	-5.46±2.86	-5.25±4.19	0.727

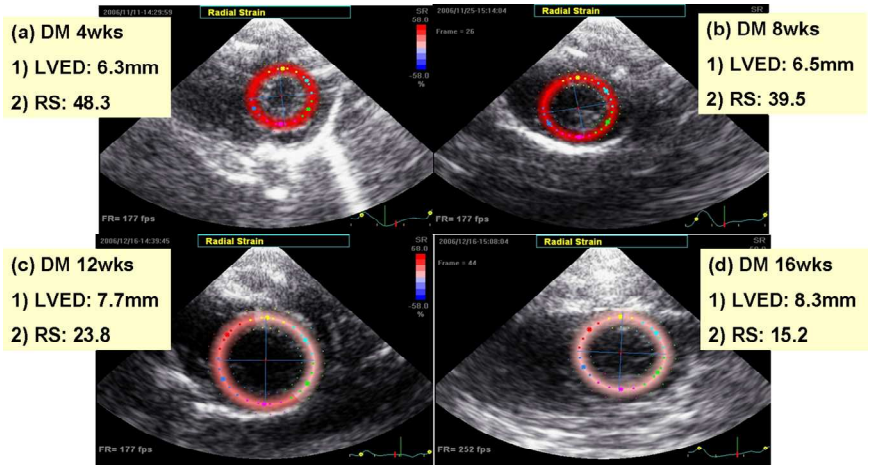

Figure 6. The change of left ventricular dimension and the systolic function in radial direction with diabetes progression. The red color means the length of myocardial movement (radial direction) during systolic phase (that is, peak radial systolic strain) and as the red is evident, the length is longer. This figure shows that the systolic function is impaired and left ventricular dimension is increased as the period is longer after diabetes induction. (DM: diabetes mellitus; LVED: left ventricular end-diastolic dimension; RS: radial strain (%))

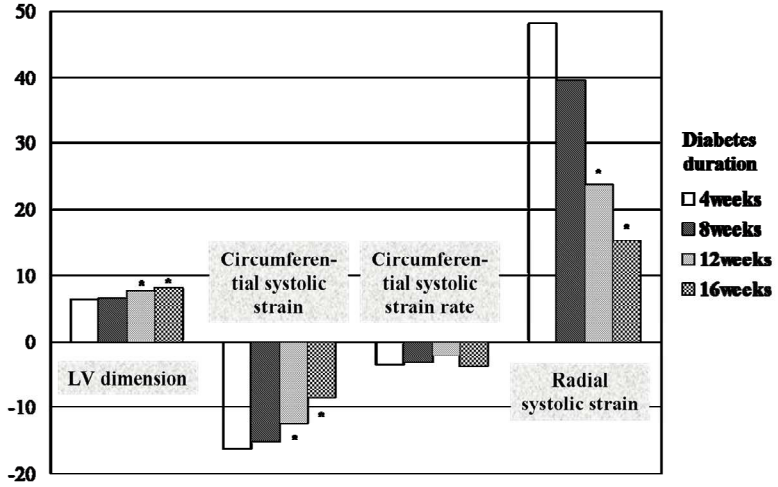

Figure 7. The sequential change of left ventricular (LV) dimension and systolic function with diabetes progression, *: $p<0.05$ significant difference compared to DM 4 weeks

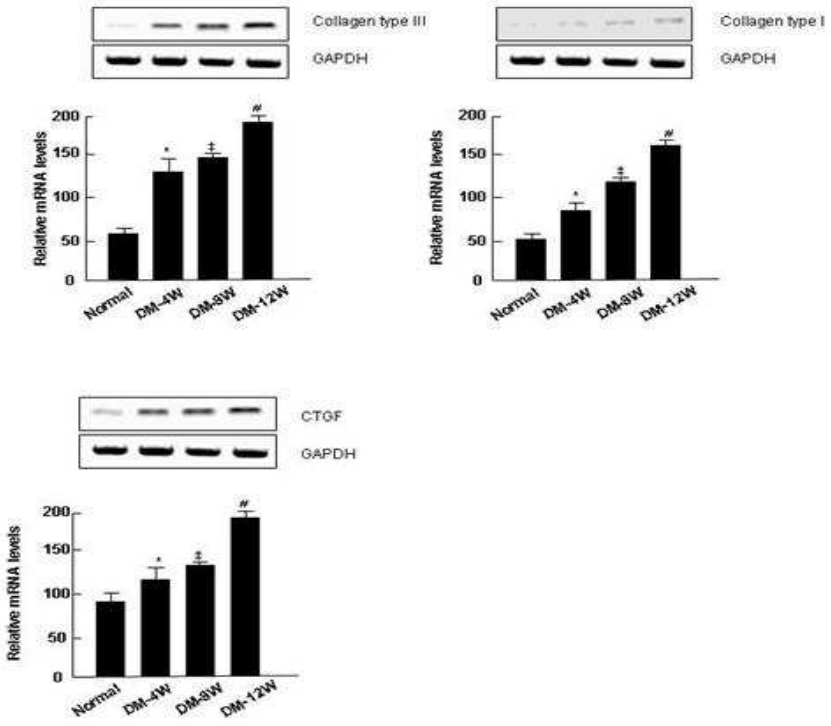

Figure 8. The change of gene expresssion related to cardiac structure in heart with diabetes progression. * p <0.05 vs Control, ‡ <0.05 vs DM 4 weeks, ## <0.05 vs DM 8 weeks

7. The functional change after alagebrium administration at diabetes rat

The myocardial functional change after alagebrium administration was analyzed through echocardiography. The LV dimension had tendency to be decreased at alagebrium group compared to control group (p=0.099). There was also improvement in radial systolic strain and circumferential strain rate with borderline statistical significance (Table 3).

Table 3. The echocardiographic parameters in alagebrium group compared to control group

	Control group (n=10)	Alagebrium group (n=10)	P value
LV dimension (mm)	8.33±0.58	7.33±0.58	0.099
Ejection fraction (%)	71.67±10.45	70.11±2.07	0.513
Circumferential strain	-8.46±2.78	-13.44±2.18	0.127
Circumferential systolic strain rate (1/s)	-3.62±0.74	-2.47±0.16	0.050
Circumferential diastolic strain rate (1/s)	2.74±0.48	2.34±0.47	0.268
Radial systolic strain	15.21±5.75	38.69±6.94	0.050
Radial systolic strain rate (1/s)	4.82±2.29	7.19±0.30	0.275
Radial diastolic strain rate (1/s)	-5.25±4.19	-5.57±1.09	0.513

IV. DISCUSSION

The present study demonstrates that alagebrium chloride (ALT-711), an AGE cross link breaker, improves myocardial systolic dysfunction and LV remodeling in a streptozocin induced diabetic rat. These effects of alagebrium are mediated by inhibition of ERK signaling pathway via reduction of intracellular ROS induced by AGE.

It has been known that diabetes led not only to marked accumulation of cardiac AGEs, but also to a significant increase in crosslinked collagen and enhanced expression of the fibrillar collagen, type III collagen. The formation of AGEs is a complex and long-lasting process. It begins with a nonenzymatic reaction between glucose and proteins, known as the browning or Maillard reaction, to form reversible early glycation products (Shiff bases). [2,3] Over a period of days or weeks, the Shiff base transforms into more stable Amadori products that tend to accumulate on proteins. This process is also reversible; however, the formation of Amadori products from Shiff bases is much faster than the reverse reaction. The formation of Shiff bases and Amadori products is proportional to glucose concentration. Therefore, the concentration of Amadori products, such as hemoglobin A1C, may be used to assess the degree of glucose control over several weeks. Amadori products formed on long-lived proteins, such as collagen, undergo further reactions to form AGEs. In contrast to their precursors, AGEs are virtually irreversible once formed. Because AGEs are stable adducts, they accumulate continuously on long-lived proteins with aging. [5,6] Furthermore, AGE related crosslinks are resistant to enzymatic degradation, which may be one of the reasons for the increase of collagen content in cardiovascular tissue with aging.

One of the most prominent consequences of increased AGE accumulation is increased collagen crosslinking in the cardiovascular tissue, with a resulting increase in myocardial and vascular stiffness, which in turn confers

considerable risk for cardiovascular morbidity and mortality. Additionally, AGEs exert adverse effects on different cell types, most notably on endothelial and smooth muscle cells. Thus, they stimulate proinflammatory mechanisms, increase production of superoxide anions, affect endothelium-mediated smooth muscle cell function, and increase oxidative stress. As shown in Figure 1 and 2, AGE and glucose level as a stimulator induced the production of ROS, a superoxide anion, in cardiomyocytes but alagebrium made an inhibitory effect on ROS formation by AGE. The result suggested that alagebrium might regulate the oxidative stress in AGE-stimulated cardiomyocytes.

AGE receptors are expressed on a wide range of cells including smooth muscle cells, macrophages, endothelial cells, and podocytes. In diabetes, it is postulated that the increase in AGEs activates AGE receptors. Moreover, there is experimental and clinical evidence that RAGE expression is increased in the blood vessels and kidneys in diabetes. Diabetes is also associated with a significant increase in left ventricular RAGE protein. [30-33] In our study, a significant increase in RAGE gene expression was detected in AGE stimulated cardiomyocytes but alagebrium suppressed the expression of RAGE. Additionally, alagebrium regulated MAPKinase system in AGE-stimulated cardiomyocytes. It has been known that ERK (extracellular signal-regulated kinase) 1/2 and AGE were implicated in the pathogenesis of diabetic complications although contribution from other signaling pathways should not be excluded at this time. As illustrated in Figure 4, alagebrium reduced the activation of ERK 1/2 by the treatment with AGE.

The increased expression of RAGE protein in the LV of diabetic animals implies a role for this receptor in mediating AGE-induced myocardial structural alterations. Furthermore, the overexpression of RAGE may influence AGE receptor–mediated events by modifying the function of the

AGE-receptor complex. This receptor may also exert direct effects on cardiac remodeling, independently of AGE ligands, by virtue of its adhesive and growth-regulating properties. [32] In this study, the expression levels of collagen type I and III involved in structure were increased but also cardiac dysfunction was developed with diabetic progression (Figure 6, 7, and 8)

Extracellular collagen matrix is an important determinant of the structural integrity and function of the cardiovascular system. Under physiologic conditions, enzymatically formed pyridinoline crosslinks connect collagen fibers and form fibrillar collagen matrix, which in the heart provides support for individual myocytes, ensuring a structural basis for their contraction and relaxation, which then translates into overall myocardial function. Similarly, the unique structural organization of collagen and elastin fibers provides both necessary firmness and distensibility for the normal functioning of the large arteries and aorta. [34] It has been shown recently that a loss of collagen support caused by increased degradation of mature collagen with replacement by newly synthesized collagen with decreased crosslinking may contribute directly to left ventricular dilatation and dysfunction. In addition to enzymatically formed pyridinoline crosslinks, collagen crosslinks may be formed nonenzymatically through the formation of AGEs. Excessive collagen crosslinking, caused by the formation of AGEs, increases cardiovascular stiffness and imparts considerable risk for cardiovascular morbidity and mortality. [35]

Treatment with alagebrium in diabetic condition completely prevented the increase in collagen III gene and protein expression but also was associated with a significant reduction in CTGF gene and protein expression assessed by RT-PCR. (Figure 5) Cardiac function was directly assessed in this study, and echocardiographic studies have shown restored cardiac contractility in diabetic model after treatment with alagebrium. (Table 3) The effect of

alagebrium on diabetic heart might result from the reduction of ROS synthesis induced by AGE. It is postulated that therapy with ALT-711 could prove useful in attenuating cardiac remodeling and in reducing the development of cardiac disease in diabetes.

V. Conclusion

Alagebrium treatment in diabetic rat improved myocardial systolic dysfunction and LV remodeling because it blocked the intracellular ROS synthesis resulting in inhibition of contractile dysfunction induced by AGE. The alagebrium reduced expression of CTGF and extracellular matrix in AGE-stimulated cardiomyocytes and this might be mediated by decreased activation of ERK (extracellular signal-regulated kinase) MAP kinase and ROS formation.

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ABSTRACT (in Korean)

1 형 당뇨 쥐 모델에서 advanced glycation end products (AGE) breaker 인 alagebrium 이 심근의 구조와 기능에 미치는 영향

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서혜선

지속적인 고혈당은 advanced glycation end products (AGE)라는 물질의 형성을 통해서 당뇨성 합병증을 유발한다. 가교제 (crosslink)의 파괴자인 3-phenacyl-4,5-dimethylthiazolium chloride (ALT-711) 즉, alagebrium chrolide라고 명명된 약제는 AGE의 상호결합을 방해함으로 인해서 이러한 합병증들을 줄일 수 있다고 알려져 있다. 그러나, AGE에 의존적인 신호전달이나 구조 분자 생리학적 복구에서 alagebrium의 효과를 고려한 평가가 많이 이루어지지 않았다. 또한 구조적·분자적 상호 연구에 관한 기능적 변화에 관하여도 거의 연구가 되지 않은 실정이다. 우리는 세포 기작에 근거를 둔 당뇨 쥐 모델에서의 구조적 변화뿐 아니라 심장초음파를 이용한 심근 기능 변화에서 alagebrium이 어떻게 효과를 나타내는지 연구하였다. AGE는 심근세

포에서 농도 의존적으로 활성산소종 (ROS)을 높였다. Alagebrium은 AGE에 의해 높아진 활성산소종을 감소시켰으며 AGE 수용체 (RAGE)의 발현을 감소시켰다. Western blot과 RT-PCR 실험에서 alagebrium은 또한 MAP kinase의 AGE로 인해 유도된 활성을 제어했고 콜라겐 성장인자(CTGF)와 세포 외 기질을 감소시켰다. 당뇨발생 이후 시간이 지날수록 심장초음파에서는 좌심실 크기가 증가됐으며 (6.33 ±0.58mm 당뇨전 vs. 8.33±0.58mm 당뇨 16주째, p=0.003), 심장근육의 반경방향으로의 수축기능을 나타내는 radial strain값은 감소하였다 (47.7±5.5% 당뇨전 vs. 15.2±5.8% 당뇨 16주째, p=0.048). 당뇨가 진행될수록 또한 콜라겐 생성은 증가되었다. 하지만 alagebrium으로 4주간 치료하였을 때 좌심실의 리모델링이 감소하였고 (8.33±0.58mm 치료전 vs. 7.33±0.58mm 치료후, p=0.099) 심근의 수축 기능장애도 호전되는 양상을 보여 radial strain 값이 치료전 15.2±5.8% 에 비해 치료후에는 38.7±6.9%로 증가되었다 (p=0.05). 결과적으로, 당뇨 쥐 심장에의 alagebrium 처리는 AGE에 의해 증가된 세포내 활성산소종 합성을 방해함으로써 좌심실 리모델링과 심근의 수축기 기능장애를 개선시켰다. 당뇨쥐 심장에서 alagebrium의 기대되는 효과는 세포 외 기질 형성의 축소와 연관되며 이는 아마도 ERK MAP kinase의 활성을 제어하기 때문일 것으로 추측된다.

핵심되는 말: advanced glycation end products (AGE), 가교제 파괴자 (ALT-711), alagebrium, 당뇨쥐 심장