

Effects of Homocysteine and Hyperglycemia on the Proliferation of Aortic Vascular Smooth Muscle Cells of Obese Type 2 Diabetes Rat

Hyung Joon Yoo^{1,*}, Sung Hoon Yu^{1,2,*}, Young Jung Cho³, Hong Woo Nam³, Dong Hyun Kang¹

¹Division of Endocrinology and Metabolism, Hallym University Kangnam Sacred Heart Hospital, Hallym University College of Medicine, Seoul, ²Division of Endocrinology and Metabolism, Hanyang University College of Medicine, Seoul, ³Department of Internal Medicine, National Medical Center, Seoul, Korea

Corresponding Author:
Hyung Joon Yoo, MD, PhD
Department of Internal
Medicine, Hallym University
Kangnam Sacred Heart Hospital,
1 Singil-ro, Yeongdeungpo-gu,
Seoul 07441, Korea

Tel: +82-2-2639-5691
Fax: +82-2-2677-9756
E-mail: hjoonyoo@gmail.com
*These authors contributed
equally to this study and should
be considered co-first authors.

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Background: The aim of this study was to investigate the role of homocysteine on the proliferation of rat aortic vascular smooth muscle cells (VSMCs) by measuring mitogen-activated protein (MAP) kinase under hyperglycemic conditions. **Methods:** Rat aortic VSMCs were isolated from Otsuka Long-Evans Tokushima Fatty and Long-Evans Tokushima Otsuka rats. VSMCs were incubated in the presence of homocysteine (1 mM) with/without PD98059 (30 μ M) and wortmannin (300 nM) for 48 hours in different concentrations of glucose (5.5, 25 mM). Proliferation was evaluated by methylthiazole tetrazolium (MTT), fluorescence-activated cell sorting (FACS), and western blot analyses. **Results:** In MTT and FACS analyses, the proliferation of VSMCs was increased by homocysteine. After PD98059 (30 μ M) and wortmannin (300 nM) treatment with homocysteine (1 mM), the increased proliferation of VSMC caused by homocysteine, decreased to control levels. In Western blot analysis, immunoexpression of phospho-p44/42 MAP kinase was significantly increased by homocysteine (1 mM). After PD98059 and wortmannin treatment, the increased immunoexpression of phospho-p44/42 MAP kinase was suppressed. **Conclusion:** These results suggest that the MAP kinase and PI3 kinase pathways are the main mechanisms involved in rat VSMC proliferation caused by homocysteine under hyperglycemic conditions.

Key Words: Homocysteine, Hyperglycemia, Vascular smooth muscle cell, Mitogen-activated protein kinase, Phosphoinositide 3-kinase

INTRODUCTION

Homocysteine is a sulfur-containing amino acid formed during methionine metabolism. In 1969, McCully first described the association between hyperhomocysteinemia and vascular disease in a patient with homocystinuria, an inborn error of homocysteine metabolism¹. Recent epidemiological studies have reported a clear association between hyperhomocysteinemia and cardiovascular mortality and atherothrombotic events. Hyperhomocysteinemia is regarded as an independent risk factor of vascular disease and insulin resistance. Insulin resistance has been implicated in the development of atherosclerosis and frequently coexists with common proatherogenic disorders such as neointimal hyperplasia². Recent studies demonstrated that hyperhomocysteinemia increased neointima formation and elastin and collagen deposition³. Although the pathophysiology linking hyperhomocysteinemia to vascular disease remains unclear, numerous stu-

dies have suggested that homocysteine is related to impaired vascular endothelial and smooth muscle cell function⁴⁻⁶.

The proliferation of vascular smooth muscle cells (VSMCs) is the most prominent hallmark of early atherosclerosis, which is also observed when premature atherosclerosis is present with diabetes^{7,8}. Okada et al.⁹ showed a significant relationship between hyperhomocysteinemia and an increased risk of coronary arteriosclerosis in patients with type 2 diabetes. Cardiovascular disease is the leading cause of mortality in patients with diabetes. Patients with severe hyperhomocysteinemia typically have neurological abnormalities or premature arteriosclerosis and develop cerebral thrombosis or myocardial infarction at approximately the age of 30 years¹⁰. A strong correlation between plasma homocysteine concentrations and mortality has been reported in patients with angiographically confirmed coronary heart disease. A meta-analysis showed that an increase of 5 mM in plasma homocysteine enhanced the risk of cardiovascular disease by 1.6 to 1.8 fold¹¹. It is now recognized that hyperhomocysteinemia

is a common risk factor for cardiovascular disease, similarly to smoking and hyperlipidemia^{2,12}.

Hyperhomocysteinemia promotes atherosclerosis primarily by injuring endothelial cells via hypomethylation and by altering hepatic lipid metabolism, thus increasing the uptake of modified-low-density lipoprotein by macrophages, leading to the accumulation of cholesterol and triglycerides in the vessel wall. The results of lipid analyses and low-density lipoprotein uptake assays suggested that hyperhomocysteinemia increases plasma total cholesterol and decreases high-density lipoprotein-cholesterol¹³. The results of many experimental studies have suggested that reactive oxygen species, nuclear factor κ B (NF κ B) activation³, and mitogen-activated protein (MAP) kinase^{14,15} are important in VSMC proliferation caused by homocysteine. However, the exact mechanisms by which homocysteine causes proliferation of VSMCs under hyperglycemic conditions remain unclear.

In this study, we investigated the role of homocysteine in the proliferation of aortic VSMCs to investigate the signaling pathway with different concentrations of glucose in an obese type 2 diabetes rat model.

MATERIALS AND METHODS

1. Study Animal

Age-matched male Otsuka Long-Evans Tokushima Fatty (OLETF) rats, a model of spontaneous noninsulin dependent diabetes mellitus, and male Long-Evans Tokushima Otsuka (LETO) rats, a nondiabetic control model of OLETF rats, were kindly provided by Otsuka Pharmaceutical Co. (Tokushima, Japan).

2. Cell Culture

VSMCs were harvested from the thoracic aorta of 12-week-old male OLETF and LETO rats by elastase and collagenase digestion as previously described^{16,17}. The cells were grown in Dulbecco's modified Eagle's medium (DMEM, Gibco-BRL, Grand Island, NY, USA) containing 10% fetal bovine serum (FBS) in a 37°C, 5% CO₂ humidified incubator. At confluence, the cells were trypsinized using 0.125% trypsin in 0.005% ethylenediaminetetraacetic acid (EDTA). Cells from passages 7–13 were used for the experiments. The cells grew in a "hill and valley" pattern, which is characteristic of VSMCs in culture¹⁸ and showed positive immunostaining with anti-smooth muscle α -actin antibodies.

3. Treatment of Cells With Reagents

Cultured VSMCs were seeded into 96-well plates (1×10^4

cells/well) in 10% FBS DMEM and incubated for 48 hours. Quiescence was induced in the cells by incubation in 0.1% FBS DMEM for 24 hours before homocysteine addition. Different concentrations of homocysteine (Sigma, St Louis, MO, USA) were added (control, 0.01, 0.1, and 1 mM). Wells containing 1 mM homocysteine were mixed or not mixed with PD98059, a MAP kinase inhibitor (30 μ M, Sigma) and wortmannin, a phosphatidylinositol-3 kinase (PI3 kinase) inhibitor (300 nM, Sigma). The cells were then incubated for an additional 48 hours in 10% FBS DMEM in the presence of different concentrations of glucose (5.5 and 25 mM).

4. Cell Proliferation Test

Cell proliferation was evaluated by the methylthiazole-tetrazolium (MTT, Sigma) assay and expressed as cell viability (%). VSMCs were incubated in the presence of homocysteine (1 mM) with/without PD98059 (30 μ M) or wortmannin (300 nM) for 48 hours with different concentrations of glucose (5.5 and 25 mM). MTT solution (5 mg/mL in phosphate-buffered saline) was then added to each well and the plates were incubated for 4 hours. The MTT formazan product was solubilized by the addition of dimethyl sulfoxide (Sigma), and absorbance was measured at 570 nm using an ELX800 (Biotek, Winooski, VT, USA).

5. Flow Cytometric Analysis of DNA Content

VSMCs were seeded in duplicate in 6-well plates. After 24 hours in DMEM containing 0.1% FBS, the medium was changed to 5% FBS. VSMCs from OLETF rats were harvested from the wells using phenol red-free trypsin-EDTA at 24 and 48 hours. The cells were immediately permeabilized and stained in the unfixed state using a solution of 0.5% wt/vol saponin and 0.1% wt/vol bovine serum albumin in phosphate-buffered saline containing 50- μ g/mL propidium iodide and 50- μ g/mL ribonuclease A. Data from the stained cell suspensions were acquired using a FACS Calibur flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA) using Cell Quest software (BD Biosciences). The resultant DNA profiles were analyzed by considering the FL2 peak area and width and using the Modfit software (Verity Software House Inc., Topsham, ME, USA) to determine the percentage of cells in each phase of the cell cycle.

6. Western Blot Analysis

The cells cultured with homocysteine (1 mM) with/without PD98059 (30 μ M) and wortmannin (300 nM) for 48 hours were washed and lysed with lysis buffer (10 mM Tris, [pH 7.4], 1 mM EDTA, 1% sodium pyrophosphate [NP]-40, 0.1 mM phenylmethylsulfonyl fluoride [PMSF], 20 nM sodium vanadate,

and 1× cocktail solution). The supernatants were analyzed using the Bradford assay (Bio-Rad, Hercules, CA, USA). Sodium dodecyl sulfate polyacrylamide gel electrophoresis was performed with a 10% resolving gel, followed by nitrocellulose membrane transfer (Bio-Rad). The membranes were blocked for 1 hour at room temperature (23°C) in blocking solution (5% skim milk in Tris buffer with Tween-20 [TBST, 200 nM Tris, 500 mM NaCl, pH 7.5, 0.05% Tween-20]), and then incubated overnight at 4°C with anti-phospho-p44/42 MAP kinase antibody (Cell Signaling Technology, Danvers, MA, USA), anti-phosphoinositide 3-kinase (PI3K) antibody (Cell Signaling Technology), and anti-NFκB antibody (Sigma). The membranes were washed with TBST (5 minutes, 3 times) and incubated with peroxidase-conjugated anti-mouse IgG antibody (Amersham Life Science, Amersham, UK). The membrane was washed and incubated using a Visualizer Western blot detection kit (Upstate, Lake Placid, NY, USA), and autoradiography was performed. Band intensity was analyzed by densitometry using Image G program.

7. Statistics

Data are expressed as the means±standard deviation. Statistical analysis was performed with Student t-test or one-way analysis of variance. Values of $p < 0.05$ were considered statistically significant.

RESULTS

1. Effects of Homocysteine on the Proliferation of VSMCs

The proliferation of VSMCs was significantly increased by

homocysteine in a dose-dependent manner in all 12-week-old rats (OLETF and LETO) in the presence of normal glucose (Fig. 1). The proliferation of VSMCs was increased by homocysteine (1 mM) in OLETF and LETO rats and at different concentrations of glucose (5.5 and 25 mM). In FACS analysis, the proliferation of VSMCs was increased after treatment with homocysteine (1 mM). Flow cytometric analysis of DNA content revealed that a significantly greater percentage of homocysteine-treated VSMCs were in the G2M phase compared to controls at 24 and 48 h (Fig. 2). After PD98059 (30 μM) and wortmannin (300 nM) treatment with homocysteine (1 mM), the increased proliferation of VSMCs caused by homocysteine, decreased to control levels in all rats and at different concentrations of glucose (OLETF rats in high glucose: homocysteine vs. homocysteine+PD98059, 180.0%±2.1% vs. 120.1%±2.0%; homocysteine vs. homocysteine+wortmannin, 180.0%±2.1% vs. 122.5%±6.4%; $p < 0.05$) (Fig. 3). Following treatment with PD98059, proliferation showed a greater decrease in VSMCs compared to controls in the MTT assay. We determined the cell numbers before and after drug treatment to investigate the cytotoxicity of PD98059. There were no changes in cell numbers between the control group and treatment group (PD98059) (Supplementary Fig. 1). Following treatment with homocysteine in 49-week-old OLETF rats (overt hyperglycemic), the proliferation of VSMCs also increased at both concentrations of glucose tested (5.5 and 25 mM).

VSMC proliferation in the presence of a high glucose concentration (25 mM) was significantly higher than that in the presence of normal glucose concentration (5.5 mM) in OLETF rats (116.2%±4.3% vs. 100.0%±8.2%, $p < 0.05$) and LETO rats (120.5%±10.3% vs. 100.0%±4.2%, $p < 0.05$). Increased VSMC

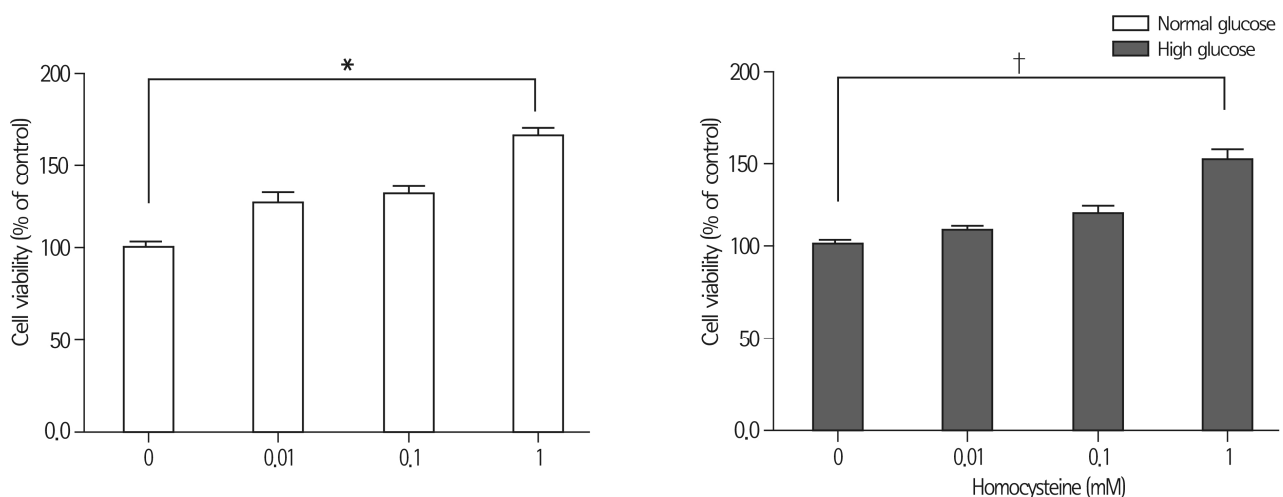


Fig. 1. Effect of homocysteine on the proliferation of vascular smooth muscle cells of Otsuka Long-Evans Tokushima Fatty rats at various concentrations. Data are expressed as the mean±standard deviation. Normal glucose, 5.5 mM; high glucose, 25 mM (n=5). * $p < 0.05$ vs. no homocysteine treatment. † $p < 0.001$ vs. no homocysteine treatment.

proliferation with homocysteine was significantly higher in the presence of high glucose than in the presence of normal glucose concentrations in OLETF rats (178.1%±7.3% vs. 155.0%±11.5%, $p < 0.05$) and LETO rats (164.5%±7.3% vs.

132.2%±8.7%, $p < 0.05$). VSMC proliferation was significantly higher in the OLETF rats compared to in LETO rats with normal glucose concentration (Fig. 3).

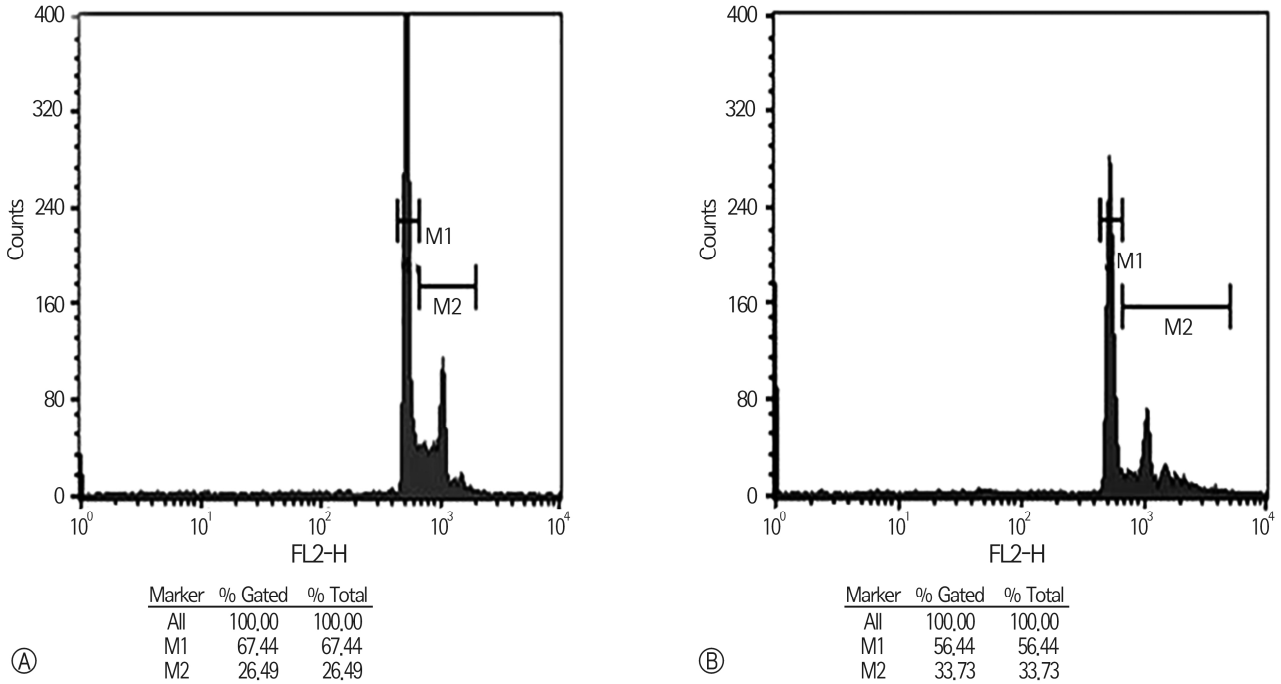


Fig. 2. Fluorescence-activated cell sorting analysis of homocysteine-treated vascular smooth muscle cells. (A) No homocysteine treatment. (B) homocysteine (1 mM) treatment.

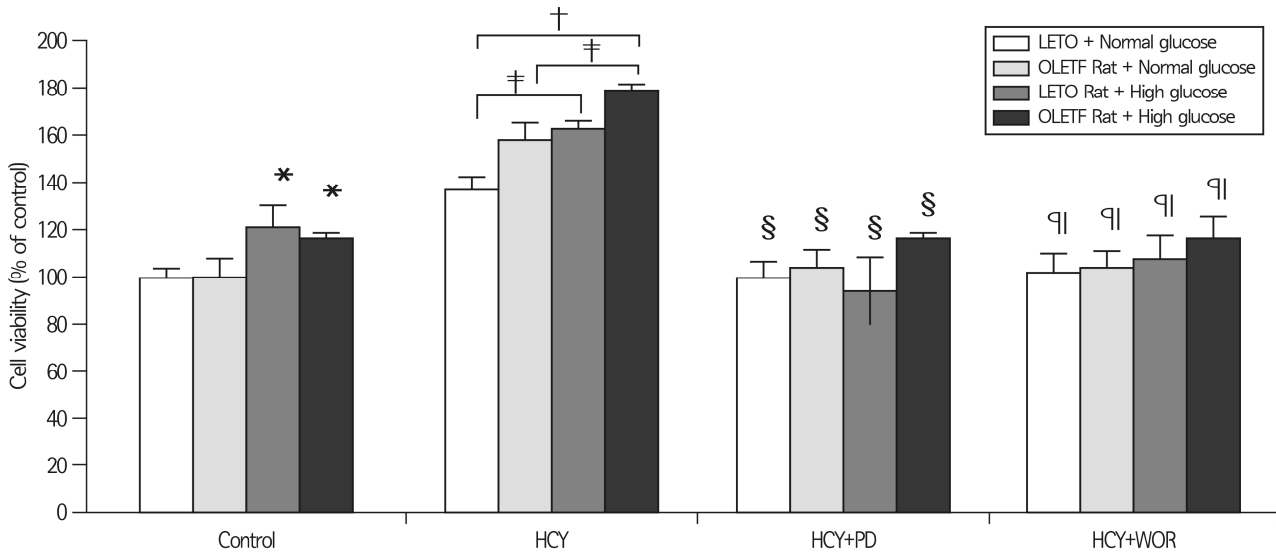


Fig. 3. Effect of homocysteine on the proliferation of vascular smooth muscle cells at different concentrations of glucose. Data are expressed as the mean±standard deviation. OLETF, Otsuka Long-Evans Tokushima Fatty; LETO, Long-Evans Tokushima Otsuka; HCY, homocysteine (1 mM); PD, PD98059 (30 μM); WOR, wortmannin (300 nM); normal glucose, 5.5 mM; high glucose, 25 mM (n=5). * $p < 0.05$ vs. normal glucose. † $p < 0.05$ vs. control. ‡ $p < 0.05$ vs. normal glucose with homocysteine. § $p < 0.05$ vs. homocysteine. ¶ $p < 0.05$ vs. homocysteine.

2. Role of MAP Kinase and PI3 Kinase Pathways on the Proliferation of VSMCs: Western Blot Analysis

Immunoexpression of phospho-p44/42 MAP kinase was significantly increased by homocysteine (0.1, 1 mM) in a dose-dependent manner among all rats at both normal and high-glucose concentrations (Fig. 4). Immunoexpression of non-phospho-p44/42 MAP kinase was not changed by homocysteine treatment (1 mM) in all rats. However, immunoexpression of phospho-p44/42 MAP kinase was significantly increased by homocysteine (1 mM) in all rats at both concentrations of glucose. After PD98059 and wortmannin treatment, the increased immunoexpression of phospho-p44/42 MAP kinase was suppressed (Fig. 5). Immunoexpression of NF κ B was not changed by homocysteine (1 mM) in OLETF rats (Fig. 6).

DISCUSSION

VSMCs play an important role in the development and

progression of atherosclerosis^{4,8}). Hyperglycemia enhances the proliferation of VSMCs, which is a critical step in the pathogenesis of atherosclerosis development¹⁹⁻²¹).

Homocysteine is the product of the combined pool of homocysteine, homocystine, mixed disulfides involving homocysteine, and homocysteine thiolactone found in the plasma of patients with hyperhomocysteinemia; approximately 5%-7% of the general population has mild hyperhomocysteinemia²²). Homocysteine is formed during methionine metabolism and is metabolized by one of 2 pathways: remethylation or transsulfuration. Hyperhomocysteinemia is caused either by a genetic abnormality of the enzymes involved in homocysteine metabolism (cystathione β -synthase deficiency) or by nutritional deficiencies in vitamin cofactors (folate, vitamin B₁₂, and vitamin B₆).

Although the exact pathophysiologic mechanism linking hyperhomocysteinemia to atherosclerosis remains unclear, numerous studies have suggested that homocysteine causes endothelial dysfunction, increases oxidative stress and lipid peroxidation, and stimulates the proliferation of VSMCs²²,

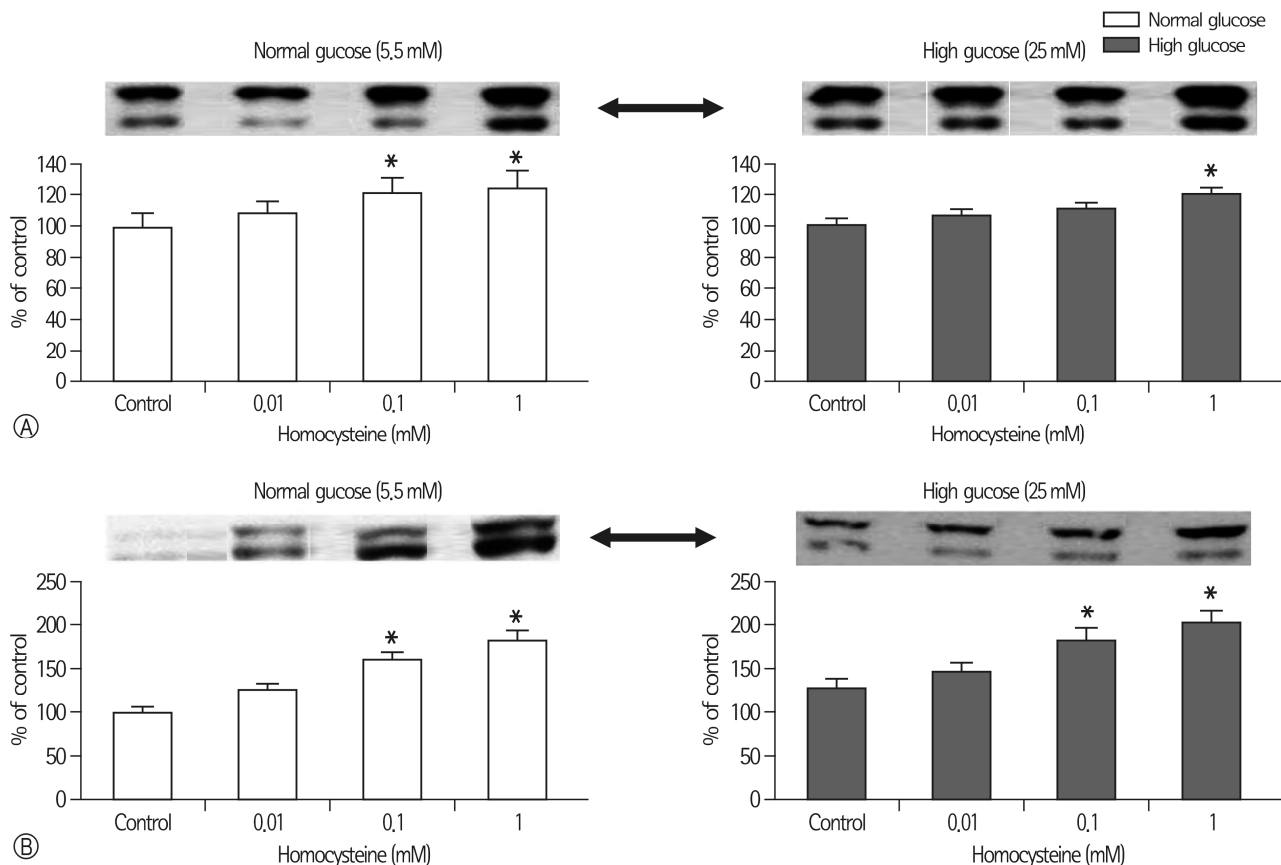


Fig. 4. (A) OLETF rat, (B) LETO rat. Enhanced effect of homocysteine on proliferation of vascular smooth muscle cells (VSMCs) through the mitogen-activated protein kinase (MAPK) signaling pathway determined by western blot analysis. Data are expressed as the mean \pm standard deviation. OLETF, Otsuka Long-Evans Tokushima Fatty; LETO, Long-Evans Tokushima Otsuka; HCY, homocysteine; \leftrightarrow , phospho-p44/42 MAP kinase antibody (42/44 kDa) (n=3). *p < 0.05 vs. control.

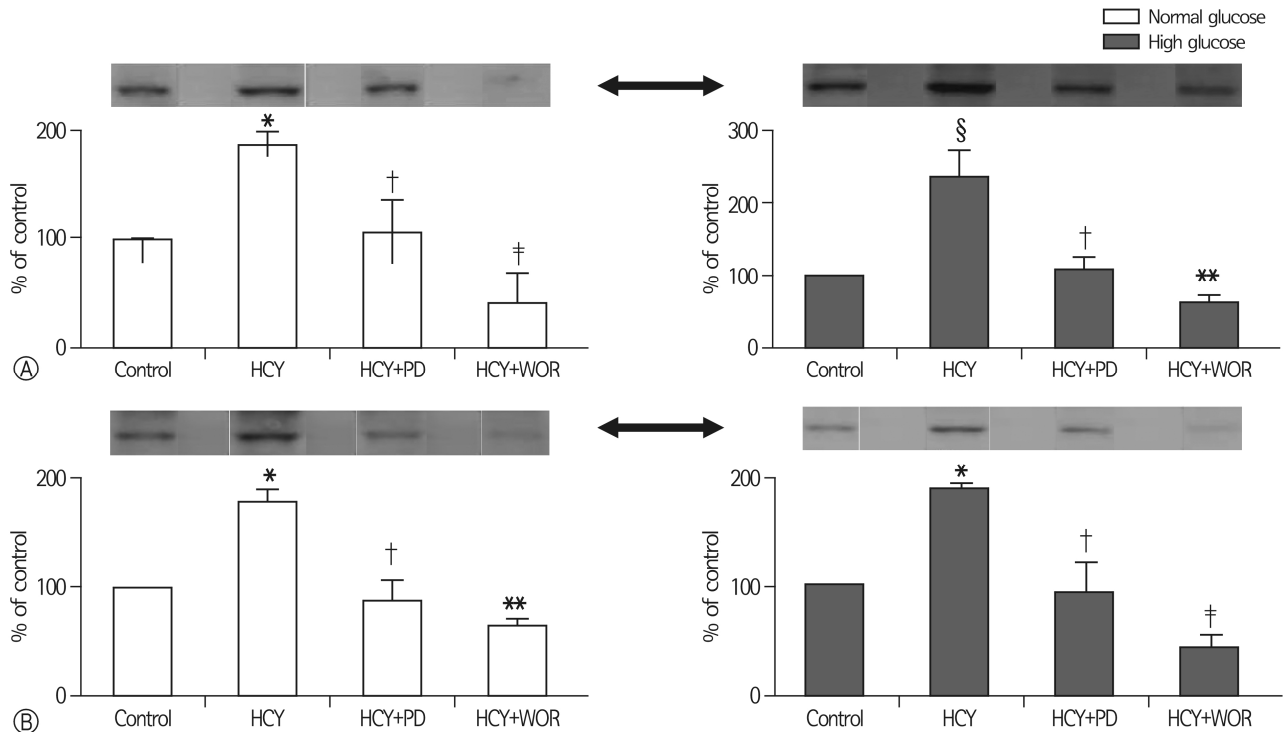


Fig. 5. (A) OLETE rat, (B) LETO rat. Role of the mitogen-activated protein (MAP) kinase pathway in the proliferation of vascular smooth muscle cells: Western blot analysis. Data are expressed as the mean \pm standard deviation. OLETF, Otsuka Long-Evans Tokushima Fatty; LETO, Long-Evans Tokushima Otsuka; HCY, homocysteine (1 mM); PD, PD98059 (30 μ M); \leftrightarrow , phospho-p44/42 MAP kinase antibody (42/44 kDa) ($n=3$). * $p<0.05$ vs. control. ** $p<0.05$ vs. homocysteine. † $p<0.05$ vs. control. ‡ $p<0.05$ vs. HCY+WO.

NF κ B activity, and monocyte chemoattractant protein-1 expression⁴).

In the past several years, many studies have reported the relationship between homocysteine and VSMC proliferation. Brown et al.²³ detected extracellular signal-regulated protein kinase 2 activation by homocysteine, and Tsai et al.²⁴ reported that homocysteine stimulates the expression of mRNA of cyclin A and D, which was shown to be related to the control of cell growth. Doronzo et al.²⁵ reported that homocysteine increases matrix metalloproteinase-2 activity in cultured human VSMCs.

The OLETF rats used in this study are an animal model for the spontaneous development of obese type 2 diabetes. Hyperglycemia and chronic complications develop during aging; by 24 weeks, OLETF rats exhibit significant diabetes based on the oral glucose tolerance test²⁶. In this study, we used both 12- and 49-week-old rats, just prior to the development of diabetes and the overt diabetes model. LETO rats were from the same colony as the OLETF rats, but they did not develop diabetes and were used as nondiabetic controls.

The results of our study showed that phospho-p44/42 MAP kinase activation was increased by homocysteine in a dose-dependent manner at normal (5.5 mM) and high (25 mM) glu-

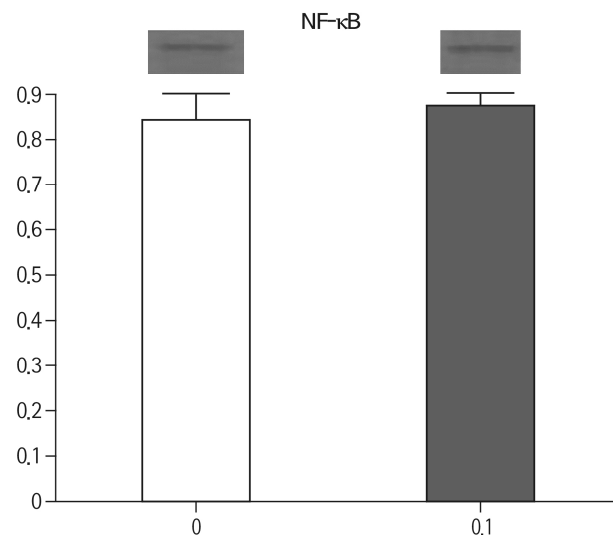


Fig. 6. Immunoeexpression of nuclear factor κ B (NF κ B) by treatment with homocysteine. HCY, homocysteine (1 mM).

cose concentrations. The increased proliferation of VSMCs and expression of phospho-p44/42 MAP kinase by homocysteine (1 mM) were suppressed by PD98059, a MAP kinase inhibitor, and wortmannin, a PI3 kinase inhibitor. There was

no difference in suppression between PD98059 and wortmannin according to the MTT assay results; however, MAP kinase activation was suppressed more significantly by PD98059 than by wortmannin, as shown by western blot analysis. Activation of phospho-p44/42 MAP kinase was partially blocked by inhibitors of PI3 kinase, and PI3 kinase was partially blocked by PD98059 (MAP kinase inhibitor). This may be because of crosstalk between the MAP kinase and PI3 kinase pathways.

Our results showed that the MAP kinase pathways play a role in the proliferation of VSMCs caused by homocysteine. In addition, the findings were similar in all rats, suggesting that genetic background does not significantly contribute to the results.

In conclusion, our results suggest that the MAP kinase and PI3 kinase pathways are the main mechanisms involved in the proliferation of VSMCs of genetically determined obese type 2 diabetes rats caused by homocysteine under hyperglycemic conditions.

Conflicts of Interest Disclosures: The researchers claim no conflicts of interest.

Acknowledgements

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Supplementary Material

Supplementary Fig. 1 can be found via <http://www.e-agmr.org/src/sm/agmr-21-78-s001.pdf>.

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