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# Insulin and nateglinide reduce monocyte adhesion to endothelial cells in Goto-Kakizaki rats exhibiting repetitive blood glucose fluctuation ☆

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**Abstract**

Epidemiological studies demonstrated the importance of postprandial hyperglycemia on the progression of atherosclerosis. However, whether treatment of postprandial hyperglycemia by insulin or insulin secretagogues has a beneficial effect on atherosclerosis has not been elucidated. To elucidate the effects of reduction of postprandial rise of blood glucose by insulin and nateglinide on monocyte adhesion to endothelial cells, we used non-obese type 2 diabetic Goto-Kakizaki (GK) rats fed twice daily, as a model of repetitive postprandial hyperglycemia. We investigated the effects of insulin injection and nateglinide administration just before each meal for 12 weeks on monocyte adhesion to endothelial cells. By setting the doses of insulin and nateglinide, both treatment significantly reduced postprandial hyperglycemia without significant reduction of $HbA_1c$. Nateglinide also reduced serum insulin level just after 1 h meal. Both nateglinide and insulin therapy reduced the number of monocytes adherent to the aortic endothelial layer. Nateglinide, but not insulin, reduced intimal thickness of the thoracic aorta. While increased serum insulin level might be regarded as a factor responsible for the progression of atherosclerosis, our data showed that treatment with pre-meal insulin or nateglinide, which reduces postprandial hyperglycemia, reduced monocyte adhesion to endothelial cells.


*Keywords:* Type 2 diabetes; Postprandial hyperglycemia; Atherosclerosis; Monocyte; Macrophage; Cardiovascular disease

The worldwide prevalence of impaired glucose tolerance (IGT) and type 2 diabetes mellitus is increasing [1]. One of the major problems faced by these patients is the excess morbidity and mortality associated with cardiovascular disease [2]. Numerous epidemiological studies showed that subjects with postprandial hyperglycemia are at increased risk of cardiovascular disease [3,4]. The α-glucosidase inhibitors are a class of drugs for diabetes that significantly reduce the postprandial rise in glucose without increasing serum insulin level. Several clinical studies showed the efficacy of acarbose, a member of α-glucosidase inhibitors, in prevention of cardiovascular diseases [5,6]. On the other hand, nateglinide is a D-phenylalanine derivative and insulinotropic agent with rapid-onset and short duration of action [7]. It is used as a mealtime insulin secretagogue in the treatment of type 2 diabetes. By reducing the postprandial blood glucose peak, nateglinide lowers the 24-h blood glucose profile and reduces the $HbA_1c$ level without increasing total insulin release [8,9]. While some data

☆ *Abbreviations:* 8-OHdG, 8-hydroxydeoxyguanosine; CS-1 fibronection, connecting segment-1; eNOS, endothelial nitric oxide synthetase; FFA, free fatty acid; GK rat, Goto-Kakizaki rat; HO-1, heme oxygenase-1; ICAM-1, intercellular adhesion molecule-1; IL, interleukin; MCP-1, monocyte chemoattractant protein-1; NEMOes, new *en face* method for optimal observation of endothelial surface; PAI-1, plasminogen activation inhibitor-1; TC, total cholesterol; TG, triglyceride; VCAM-1, vascular cell adhesion molecule-1; VLA-4 fibronection, very late-acting antigen-4.

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demonstrated that the increase in serum insulin level might promote the progression of atherosclerosis [10,11], it has not been elucidated whether treatment with insulin or insulin secretagogues to reduce postprandial hyperglycemia prevents the progression of atherosclerosis.

Recently, we established a new *en face* method for optimal observation of endothelial surface (NEMOes), which is suitable to quantitate the number of monocytes that adhere to rat thoracic aorta after immunostaining of the monocyte/macrophage specific protein, CD68 [12,13]. Using NEMOes, we investigated endothelial function in the Goto-Kakizaki (GK) rat, a genetic non-obese type 2 diabetes rat, fed twice daily as a model of repetitive postprandial hyperglycemia [14]. Our results demonstrated that repetitive postprandial hyperglycemia *per se* induced monocyte adhesion to endothelial cells compared with persistent hyperglycemia [15]. In addition, we reported that α-glucosidase inhibitors could efficiently reduce daily blood glucose fluctuation and reduce monocyte adhesion to endothelial cells and intimal thickening of thoracic aorta [16].

The present study is an extension to our earlier investigations [12,13,15,16] and was designed to determine the effects of nateglinide and preprandial injection of insulin (to reduce postprandial hyperglycemia) on monocyte adhesion to endothelial cells and intimal thickening. In this study, to elucidate the effect of postprandial hyperglycemia and its suppression, we used the doses of insulin and nateglinide to reduce postprandial hyperglycemia alone without significant reduction of $HbA_1c$.

## Materials and methods

*Animals*. The study protocol was reviewed and approved by the Animal Care and Use Committee of Juntendo University. Male GK rats ($n = 18$) obtained from Charles River Japan (Yokohama, Japan) at the age of 6 or 7 weeks were housed in a polycarbonate cage with a wooden chip mat on the floor. Water was available *ad libitum* for all rats. Standard CRF-1 chow (22.6% protein, 53.8% carbohydrate, 5.6% fat, 6.6% mineral and vitamin mixture, and 3.3% fiber, total: 356 kcal/100 g, Charles River Japan, Yokohama, Japan) was used in this study. The animal room was kept on a 12-h light/dark cycle (7:00 AM–7:00 PM/dark, 7:00 PM–7:00 AM/light), at a constant temperature (22 ± 1 °C) and a relative humidity of 55 ± 5% throughout the experimental period.

*Experimental design*. All rats ($n = 18$) were trained to consume the diet chow during a 1-h period, twice a day (9:00 AM–10:00 AM and 3:00 PM–4:00 PM). All rats were fed the standard diet (Fig. 1). The animals were acclimatized to laboratory conditions for 2 weeks (from at the age of 8 to 10 weeks). At the age of 10 weeks, the rats were divided into three groups and treated with either vehicle alone (0.5% methylcellulose, $n = 6$, control group), nateglinide (50 mg/kg, $n = 6$, nateglinide group) or insulin (3.6 nmol/kg, $n = 6$, insulin group). Methylcellulose and nateglinide were provided by oral gavage, and insulin was injected subcutaneously twice daily just before each meal. Body weight was measured at the age of 10, 13, 16, 19, and 22 weeks in each group (Table 1).

*Laboratory data*. Blood samples were taken from 16-week-old ($n = 6$ each) rats in each group from tail vein 17 h after fasting. The plasma glucose level was measured by the glucose oxidase method, using Glutest sensor (Sanwa Kagaku, Nagoya, Japan). Measurements of glycated hemoglobin ($HbA_1c$), total cholesterol (TC), free fatty acid (FFA), and triglyceride (TG) were outsourced to a private laboratory (SRL Co., Tachikawa, Japan). Daily blood glucose level was measured at the age of 21 weeks in each group ($n = 6$ each). Simultaneously, blood samples were taken from the tail vein before and just after finishing first diet. These samples were used for measuring serum insulin concentration using the enzyme-linked immunosorbent assay (ELISA) insulin kit (Morinaga Co., Yokohama, Japan). Urine 8-hydroxydeoxyguanosine (8-OHdG) was measured by the 8-OHdG Check (Nikken Seil Co., Shizuoka, Japan) using urine samples after 17 h fasting at the age of 22 weeks.

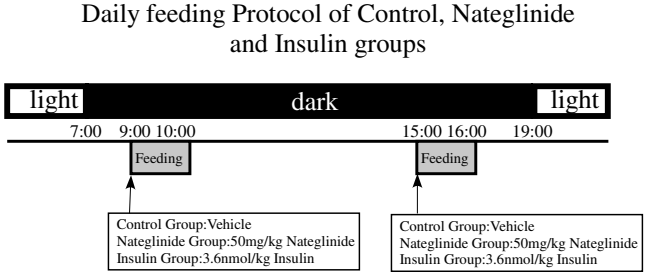


Fig. 1. Daily feeding schedule and drug intervention. Restricted diet protocol was started at the age of 8 weeks in all rats ($n = 18$). At 10 weeks of age, rats were separated into three groups, the vehicle administered group (control group; $n = 6$), nateglinide administered group (nateglinide group; $n = 6$), and insulin administered group (insulin group; $n = 6$). Daily feeding and drug administration pattern was continued until 22 weeks of age.

Table 1
Comparison of serial changes in body weight (g) in the three treatment groups

| | Age (weeks) | | | | |
|---|---|---|---|---|---|
| | 10 | 13 | 16 | 19 | 22 |
| Control group | 193 ± 4 | 254 ± 4 | 276 ± 3 | 291 ± 6 | 310 ± 9 |
| Nateglinide group | 194 ± 4 | 262 ± 8 | 280 ± 10 | 297 ± 10 | 310 ± 10 |
| Insulin group | 194 ± 3 | 263 ± 4 | 290 ± 5 | 318 ± 6 | 334 ± 8 |

Data are expressed as means ± SEM ($n = 6$). No statistical differences were observed among the groups.

*NEMOes*. Monocyte adhesion to the wall of the thoracic aorta was quantitated by NEMOes as described previously [12] in rats of each group at the age of 22 weeks ($n = 6$ each). Briefly, rats were perfused with normal saline followed by 10% buffered formalin. After fixation, the aorta was divided into 8–12 mm long segments. Each segment was then placed in 0.05% hydrogen peroxidase in methanol for 20 min at room temperature. These specimens were incubated with the mouse anti-rat CD68 antibody (Serotec, Raleigh, NC), diluted 1:100 in PBS for 60 min at 37 °C. Next, the specimens were placed with biotinylated anti-mouse IgG for 30 min at room temperature, followed by reaction with horseradish peroxidase-conjugated streptavidin with the aid of an LSAB2 kit (Dako, Glostrup, Denmark). Staining was completed after incubation with a substrate-chromogen solution. The segments were then cut open longitudinally along the ventral side with scissors. Each specimen was simply placed on a slide glass with the intimal side up and covered with a coverslip by surface tension. Specimens were viewed under a microscope (E800; Nikon, Tokyo) connected to an XYZ controller and a digital camera (Media Cybernetics Inc., Silver Spring, MD). Pictures were captured at various focal lengths with an automatically regulated Z-stepper and the clearest images were selected automatically to produce a composite image of the whole thoracic aorta by Image-Pro4.5J (Planetron Co., Tokyo). For precise quantitation of the number of monocytes adherent to the endothelium, we counted separately the number of CD68-immunopositive cells around the opening of intercostal arteries in each aorta (1400 × 1000 μm). The cell density in each area was calculated as the cell count divided by the total area by examiners blinded to the treatment regimen.

*Measurement of intimal thickness of the aorta*. To measure the intimal area of the thoracic aorta, four cross-sections of each aorta spaced at

approximately 4-mm intervals were stained with hematoxylin and eosin. The cross-sectional intimal areas of a lesion in a given photomicrograph were measured with an image analysis software (Image-Pro4.5J, Planetron Co., Tokyo) as described previously [15]. The average intimal area was then calculated for each aorta. The aorta of 22-week-old rats in each group ($n = 6$, respectively) was analyzed.

*Statistical analysis.* All data were expressed as means ± SEM. All statistical analyses were performed with SPSS Version 11 (SPSS Inc., Chicago, IL). One-way ANOVA and post-hoc test were used to compare groups. A $p$ value less than 0.05 was considered significant.

## Results

### *Laboratory findings*

Both food intake and body weight during the study period were not different among the group (data not shown and Table 1). The daily blood glucose profile showed postprandial spikes in blood glucose concentration in the control group (Fig. 2a). Treatment with nateglinide and insulin significantly reduced the postprandial rise in blood glucose level without affecting blood glucose level at other timings. Treatment of nateglinide and insulin reduced $HbA_1c$ very modestly. The differences of $HbA_1c$ among the groups were not statistically different (Table 2). On the other hand, although nateglinide is an insulin secretagogue, it reduced insulin level just 1 h after feeding (Fig. 2b). This is probably due to the rapid action of nateglinide. Injection of insulin resulted in a rise in postprandial serum insulin level compared with nateglinide, however, which is comparable with control group.

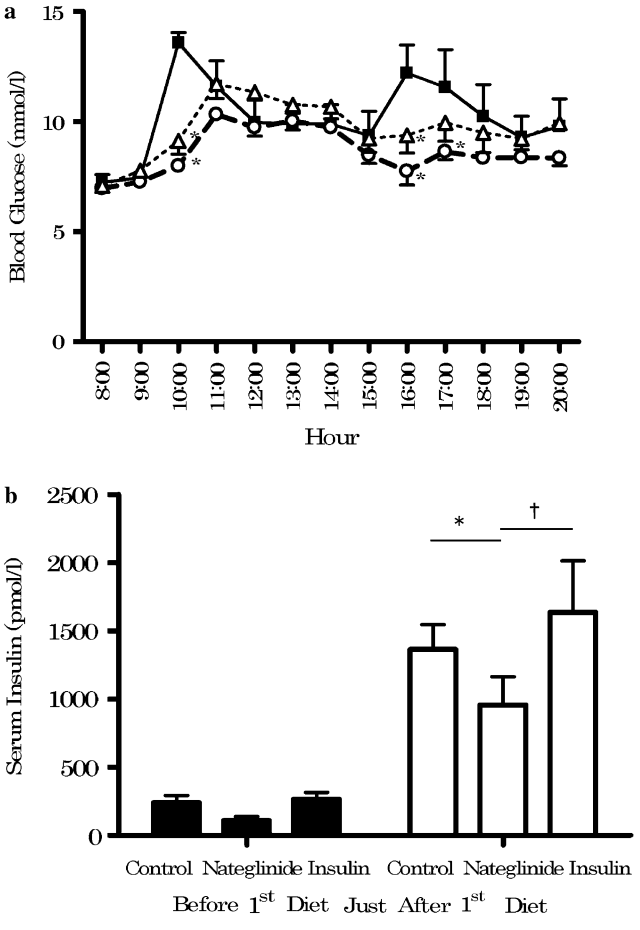


Fig. 2. Daily blood glucose profile of each group. (a) Mean blood glucose hourly profiles in control group (filled square), nateglinide group (open circle), and insulin group (open triangle) at the age of 21 weeks ($n = 5$ each). (b) Serum insulin concentrations before and 1 h after 1st diet in each group. Data are means ± SEM ($n = 5$ in each group). $^{*}p < 0.05$ compared with control group, $^{\dagger}p < 0.05$ compared with nateglinide group.

Table 2
Laboratory tests in each treatment group

| | Control | Nateglinide | Insulin |
|---|---|---|---|
| TC (mmol/l) | 3.05 ± 0.06 | 2.92 ± 0.09 | 3.15 ± 0.05 [a] |
| TG (mmol/l) | 0.66 ± 0.05 | 0.85 ± 0.11 | 1.02 ± 0.07 |
| FFA (mEq/l) | 1.00 ± 0.08 | 1.13 ± 0.09 | 0.94 ± 0.05 |
| $HbA_1c$ (%) | 3.95 ± 0.06 | 3.82 ± 0.05 | 3.85 ± 0.08 |
| Urine 8-OHdG (nmol/g Cr) | 14.8 ± 2.2 | 14.9 ± 4.1 | 16.2 ± 1.1 |

Blood data are obtained at the age of 16 weeks. Urine data are obtained at the age of 22 weeks. Data are expressed as means ± SEM ($n = 6$). No statistical differences were observed among the groups except where marked. 8-OHdG, 8-hydroxydeoxyguanosine; FFA, free fatty acid; TC, total cholesterol; TG, triglyceride.

[a] $p < 0.05\%$ compared with the nateglinide group.

Regarding the effect of each treatment on lipid profile, insulin nor nateglinide did alter the lipid profile significantly compared with control group (Table 2). Urine levels of 8-OHdG, a marker of systemic oxidative stress, were comparable among the groups (Table 2).

### *Nateglinide and insulin reduce monocyte adhesion to endothelial cells*

We counted the number of monocytes attached to the aortic endothelium after immunohistochemical staining with anti-rat CD68 antibody. Compared with the control group, the mean densities of monocytes attached to the endothelium were significantly reduced in both nateglinide and insulin groups (Fig. 3).

### *Nateglinide reduces intimal thickening of the aorta*

As shown in Fig. 4, we frequently observed cells in the intima of the aorta of GK rats at the age of 22 weeks near the monocyte adhesion area. Most of the cells located in this region were α-smooth muscle actin (α-SMA)-positive and Oil Red O stain-negative (data not shown) as demonstrated previously [15]. We measured the intimal thickness area in each group at the age of 22 weeks. Nateglinide treatment, but not that of insulin, significantly reduced intimal thickening (Fig. 4).

### *Monocyte adhesion to endothelial cells does not correlate with intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) mRNA expression levels*

To search for molecules involved in the reduced adhesion of monocytes to endothelial cells following nateglinide

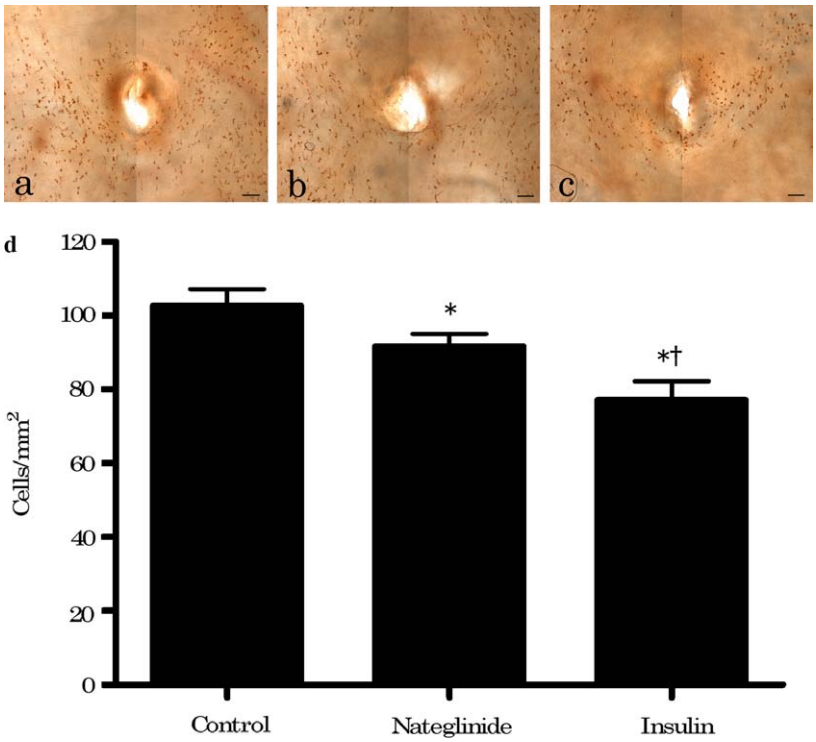


Fig. 3. Monocyte adhesion to the endothelium assessed by NEMOes. (a–c) Representative en face view of immunohistochemical staining with anti-CD 68 antibody at the age of 22 weeks from control group (a), nateglinide group (b), and insulin group (c) each group (bar = 20 μm). (d) Density of adherent CD68-positive cells on endothelial cells in each group. Data are means ± SEM. $^{*}p < 0.05$ compared with the control group, $^{\dagger}p < 0.05$, compared with the nateglinide group.

and insulin treatment, we measured the expression levels of several factors using RNAs isolated from the abdominal aorta of each group (Fig. 5). While it is widely recognized that VCAM-1, ICAM-1 play major roles in monocyte adhesion to endothelial cells, we could not find a correlation between monocyte adhesion to endothelial cells and the expression levels of these two molecules. In addition, we also investigated the expression levels of other adhesion-related molecules (connecting segment-1 [CS-1], fibronection, very late-acting antigen-4 [VLA-4]), chemokines and cytokines (monocyte chemoattractant protein-1 [MCP-1], interleukin [IL]-6, and 8), oxidative stress marker (heme oxygenase-1 [HO-1]), plasminogen activation inhibitor-1 (PAI-1), and endothelial nitric oxide synthetase (eNOS). However, the expression levels of these molecules did not correlate with the reduced number of monocyte adhesion to endothelial cells treated by nateglinide and insulin.

## Discussion

Recently, we investigated the effect of glucose fluctuation on monocyte adhesion to vascular endothelial cells, by comparing the effects of persistent hyperglycemia with those of small repetitive fluctuations in blood glucose concentrations [15]. Our results demonstrated that fluctuation in blood glucose concentrations elicited markedly higher monocyte adhesion to the endothelium compared with persistent hyperglycemia. Furthermore, prevention of fluctuations in blood glucose concentrations by phloridzin [15] and an α-glucosidase inhibitor [16] efficiently reduced monocyte adhesion to endothelial cells. These agents decrease glucose fluctuation and reduce insulin level. Thus, in this study, we investigated whether treatment with insulin or an insulin secretagogue to reduce postprandial hyperglycemia reduces monocyte adhesion to endothelial cells. Our study demonstrated that monocyte adhesion to endothelial cells is reduced by both insulin and nateglinide treatment.

In our previous studies, we used phrolidzine and acarbose to reduce postprandial hyperglycemia in the same model used in the present study [15,16]. We observed significant decreases in $HbA_1c$ level in phrolidzine (13%) and acarbose (16%) [15,16]. On the other hand, in the present study, we used the doses of nateglinide and insulin that did not result in the significant decrease in $HbA_1c$ level. Thus, the dose used in this study is more appropriate to observe the effect of glucose fluctuation *per se* on monocyte adhesion to the endothelium without the consideration of $HbA_1c$ level. Probably reflecting the degree of the reduction of glucose fluctuation, the degree of reduction of monocyte adhesion in this study was smaller compared with phrolidzine and acarbose treatment [15,16]. Thus, our study indicates that the level of suppression of postprandial glucose level is one of the major determinants of monocyte adhesion to the endothelium.

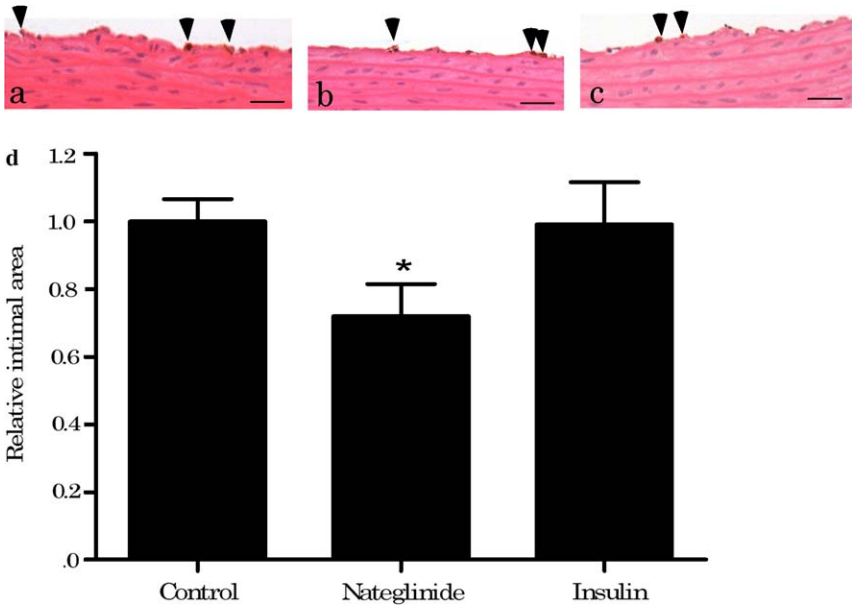


Fig. 4. Relative intimal thickening in the thoracic aorta at the age of 22 weeks. (a–c) Images of cross-sections of the thoracic artery after whole-mount staining with anti-CD68 antibody, followed by staining with hematoxylin and eosin stain from control group (a), nateglinide group (b), and insulin group (c) (bar = 5 μm). Arrowheads indicate adherent monocytes near the area of intimal thickening. (d) Relative intimal thickening was calculated by setting as 1 the value of control group. Data are means ± SEM ($n = 5$ each). $^{*}p < 0.05$ compared with the control group. $^{\dagger}p < 0.05$ compared with the nateglinide group.

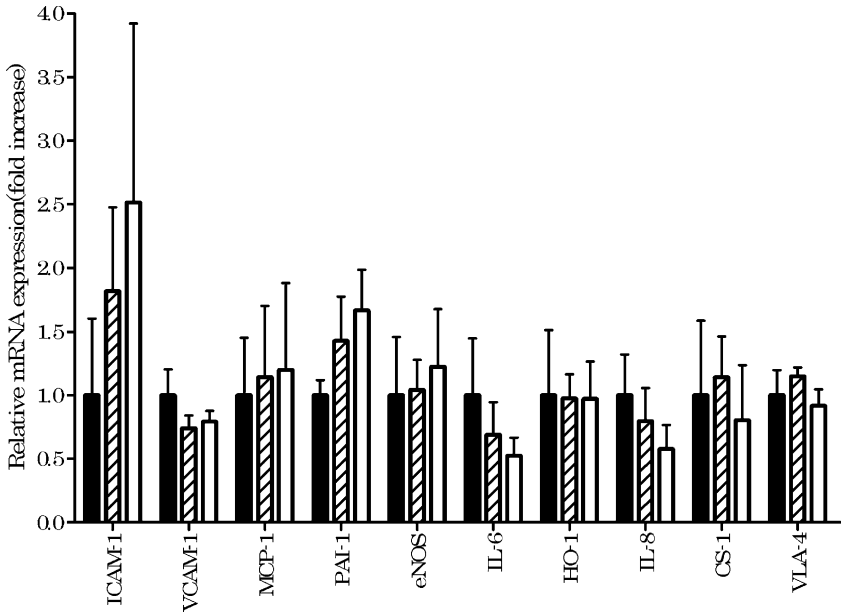


Fig. 5. Arterial gene expression. Results of quantitative RT-PCR using RNA extracted from abdominal aorta of control group (filled bar), nateglinide group (oblique bar), and insulin group (open bar) at the age of 22 weeks. The relative mRNA level was calculated with the level of control group set at 1. Data are means ± SEM of at least five rats in each group. There were no significant differences among the groups with regard to the factors investigated. CS-1 fibronection, connecting segment-1; eNOS, endothelial nitric oxide synthetase; HO-1, heme oxygenase-1; ICAM-1, intercellular adhesion molecule-1; IL, interleukin; MCP-1, monocyte chemoattractant protein-1; PAI-1, plasminogen activation inhibitor-1; VCAM-1, vascular cell adhesion molecule-1; VLA-4 fibronection, very late-acting antigen-4.

Epidemiological studies have shown the association between hyperinsulinemia with insulin resistance and the onset of cardiovascular disease [10,11]. These are partly regarded independent of other accompanying risk factors such as hypertriglyceridemia, low HDL concentration, and hypertension. Regarding the effect of insulin on monocyte adhesion to endothelial cells, it was reported that insulin promotes VCAM-1 expression in cultured endothelial cells [17], and consequently enhances monocyte adhesion to cultured endothelial cells. On the other hand, our data

demonstrated that the administration of insulin or nateglinide reduced postprandial hyperglycemia *in vivo* and in turn reduced monocyte adhesion to endothelial cells. Both insulin and nateglinide treatment did not result in significant increase in VCAM-1 expression level. Even though increases in insulin concentrations might enhance monocyte adhesion to endothelial cells, insulin or nateglinide administration just before each meal did not result in persistent hyperinsulinemia. Thus, it is possible that the beneficial effect of reduced postprandial hyperglycemia is superior to the deleterious effect of transient rise in insulin level on monocyte adhesion. Alternatively, it is possible that increased insulin levels do not induce monocyte adhesion to endothelial cells *in vivo*, not *in vitro*.

Hyperinsulinemia may increase the production of plasminogen activator inhibitor-1 [18] and various growth factors and cytokines through the activation of MAP kinase [17,19], which may result in the acceleration of smooth muscle cell turnover. However, it is still unclear whether hyperinsulinemia could promote early stage atherosclerosis. In the present study, nateglinide decreased postprandial hyperglycemia and prevented prolonged hyperinsulinemia compared with insulin treatment. Previously we demonstrated that nateglinide enhances rapid intrinsic insulin secretion, thus, efficiently reduces postprandial hyperglycemia [7]. Our present data showed that nateglinide, which prevents prolonged hyperinsulinemia, could reduce intimal thickening. However, insulin, which resulted in postprandial hyperinsulinemia, could not reduce intimal thickening, even though the level of reduction of postprandial hyperglycemia was similar. Thus, it is possible that prolonged hyperinsulinemia may be a significant determinant of intimal thickening, which cancels the beneficial effect of the reduction of postprandial hyperglycemia.

An important component of monocyte adhesion to endothelial cells is the activation and upregulation of adhesion molecules on the endothelial surface. Whereas several studies reported the importance of ICAM-1 and VCAM-1 as adhesion molecules, no significant changes in mRNA expression of ICAM-1 and VCAM-1 were observed in the present study. These findings are similar to those reported in our previous study [15]. It is possible that changes in the expression of several proteins in monocytes, structural changes, or changes in cell surface expression of adhesion molecules without changes in mRNA expression are involved in this process. Thus, further assessment is needed to complete our understanding of this process.

In conclusion, we have demonstrated that nateglinide suppressed monocyte adhesion to endothelial cells and intimal thickening in our model of non-obese type 2 diabetes characterized by fluctuation of blood glucose level. Our findings suggest that nateglinide protects against the development of cardiovascular disease.

## Duality of interest

The authors declare no duality of interest.

## Acknowledgments

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