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RESEARCH ARTICLE

The antidiabetic agent, gliclazide, reduces high insulin–enhanced neutrophil-transendothelial migration through direct effects on the endothelium

Masahiro Okouchi*
Naotsuka Okayama
Hitoshi Omi
Kenro Imaeda
Tatsuya Fukutomi
Atsushi Nakamura
Makoto Itoh

Department of Internal Medicine and Bioregulation, Nagoya City University Graduate School of Medical Sciences, Nagoya, Japan

*Correspondence to:
Masahiro Okouchi, Department of Internal Medicine and Bioregulation, Nagoya City University Graduate School of Medical Sciences, 1 Kawasumi, Mizuho-cho, Mizuho-ku, Nagoya 467–8601, Japan. E-mail: okochi@med.nagoya-cu.ac.jp

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Abstract

Background and aim Many lines of evidence indicate that hyperinsulinemia might be associated with coronary athrosclerosis, and, currently, there are no effective strategies for preventing this. We previously reported that high insulin enhances neutrophil-transendothelial migration, a process that involves increased surface presentation of platelet endothelial cell adhesion molecule-1 (PECAM-1) through a mitogen-activated protein (MAP) kinase–dependent event. In this current study, we examined if antidiabetic agents, especially K_{ATP} channel blockers, might similarly protect against the leukocyte-endothelial cell interactions enhanced by high insulin.

Methods Neutrophils transmigration across umbilical vein endothelial cells (in high insulin medium) with or without K_{ATP} channel blockers was performed. Neutrophil migration was quantified by measuring myeloperoxidase, and surface expression of endothelial PECAM-1 was examined using cell-surface enzyme immunoassay.

Results Neutrophil-transendothelial migration and PECAM-1 expression were enhanced by insulin (100 μU/mL, 24 h) and were attenuated by gliclazide (20 μM), but not by other K_{ATP} channel blockers (glibenclamide, nateglinide, and glimepiride). Neutrophil migration and PECAM-1 expression were also increased by the mitogen-activated protein (MAP) kinase activator, anisomycin (1 μM), and also attenuated by gliclazide. Nitric oxide (NO) synthase inhibitors did not modify either gliclazide effect.

Conclusions Our results suggest that the K_{ATP} channel blocker, gliclazide, blocks high insulin–mediated neutrophil migration and PECAM-1 expression. These gliclazide effects may be mediated through the inhibition of MAP kinase activation and are unrelated to NO production. Copyright © 2004 John Wiley & Sons, Ltd.

Keywords insulin resistance; hyperinsulinemia; diabetes; atherosclerosis; vascular inflammation

Introduction

There is increasing evidence that diabetes mellitus is associated with accelerated atherosclerosis [1,2]. In addition, there has been an expansion in the clinical and experimental evidence linking hyperinsulinemia and cardiovascular disease and atherosclerosis [3–5]. The United Kingdom Prospective Diabetes

Study (UKPDS) and related studies indicate that intensive control of blood glucose is effective in preventing and slowing the progression of microvascular complications in diabetic patients [6–8]. However, since effective treatments for atherosclerotic diseases, at least in part, associated with hyperinsulinemia are not available, it is important to find out what treatments are effective in preventing them. In this study, we focused on the effects of antidiabetic agents, especially K_{ATP} channel blockers, on insulin-mediated neutrophil-transendothelial migration, an event that may contribute to the atherogenesis.

Vascular inflammation is thought to potentially initiate atherogenesis in both acute myocardial infarction (AMI) and stroke, both of which are more common and serious in diabetic patients [9]. A critical and early event in vascular inflammation that may be related to atherogenesis is the extravasation of leukocyte across the endothelial layer. Among several classes of leukocytes, the neutrophils are among the first immune cells to arrive at sites of inflammation, and may play an important role in triggering later stages of inflammation [10]. Further, there is increasing evidence that supports a relationship between neutrophil migration, and both the progress and disruption of the atherosclerotic plaque [11–14]. We previously reported that high insulin enhances neutrophil-transendothelial migration and might be related to the presentation PECAM-1 (platelet endothelial cell adhesion molecule-1) on the endothelium, both events being triggered through activation of the mitogen-activated protein (MAP) kinase [15]. In our present study, we investigated which K_{ATP} channel blockers protect against inflammatory events mediated by high insulin (neutrophil-transendothelial migration and the endothelial PECAM-1 expression). We also evaluated intracellular mechanisms for the action of K_{ATP} channels blocker(s) in terms of the involvement of MAP kinase and nitric oxide (NO) metabolism, since endothelial dysfunction is often associated with decreased formation of NO [16,17]. Endothelial NO dysfunction has been protected against by several compounds, including statins, angiotensin-converting enzyme inhibitors, and angiotensin II receptor antagonists [18,19]. Therefore, we also examined the relationship between the protective actions of the K_{ATP} channels blocker(s) and NO release from endothelium.

Materials and methods

This study was carried out in accordance with the Declaration of Helsinki as revised in 2000.

Cell culture

Human umbilical vein endothelial cells (HUVEC; Kurabo, Osaka, Japan) were cultured at 37 °C in a 100% humidified atmosphere containing 5% CO_2 in CS-C medium (45% Dulbecco's modified eagle's medium, 45% Ham's F12 medium, 10% fetal bovine serum, 10-ng/mL acidic fibroblast growth factor, 50-U/mL heparin, and 15-mM HEPES (Cell Systems, Kirkland, WA)) containing antibiotic-antimycotic (Gibco BRL, Grand Island, NY) in collagen I–coated plates.

Neutrophil isolation

After informed consent, human neutrophilic polymorphonuclear leukocytes (PMN) were isolated from venous blood of healthy volunteers using dextran sedimentation and gradient separation on Histopaque 1077 (Sigma, St. Lois, MO). This procedure yields a PMN population that is 95 to 98% viable (trypan blue exclusion), with its purity accounting for 98% (acetic acid–crystal violet staining).

Assay of neutrophil transmigration across HUVEC

Neutrophil-transendothelial migration was assayed, as reported previously [15]. For transmigration experiments, HUVEC were cultured on human fibronectin-coated cell culture inserts (3.0-μm pore size; Becton Dickinson, Bedford, MA) in 24-well plates. They were incubated for 24 h at 37 °C with or without human regular insulin (Novolin R, Novo-Nordisk, Bagsvaard, Denmark) and/or various chemical agents. The surface area of each insert was 0.33 cm^2, containing approximately 16 000 endothelial cells. After treatment, culture medium in both the inserts and wells was replaced with Hank's balanced salt solution (HBSS, Gibco BRL), and 1×10^5 neutrophils were added to each upper compartment (insert) and allowed to migrate across HUVEC monolayers to the lower compartment (well) for 1 h at 37 °C. To stimulate neutrophil migration, a chemotactic peptide, formyl-methionyl-leucyl-phenylalanine (10^{-7} M, Sigma), was added to the lower compartments before adding neutrophils. Neutrophil migration was stopped by removing the inserts from the wells. The 24-well plates were centrifuged at 1500 rpm for 5 min and the migrated neutrophils in the wells were quantified with a myeloperoxidase (MPO) assay described previously [15], whereby the H_2O_2-dependent oxidation of 3,3′,5,5′-tetramethylbenzidine (Sigma) was measured. Neutrophil migration was expressed as the ratio of MPO activity of migrated neutrophils to that of the total neutrophils (1×10^5 cells).

Assay of surface expression of endothelial PECAM-1

Surface expression of endothelial PECAM-1 was measured as reported previously [15]. Briefly, after treatment of cells for 24 h with or without insulin in fibronectin-coated 24-well plates with various test agents, cells were fixed for 10 min at room temperature with 1% paraformaldehyde in PBS. After three washes with 1-mL HBSS/PBS (1 : 1), cells were incubated for 60 min

at 37 °C with an anti-PECAM-1 antibody (Ab) (primary antibody; diluted 1 : 100; Sigma) in HBSS/PBS containing 5% fetal calf serum (FCS). Cells were washed twice with 0.5-mL HBSS/PBS and incubated for 60 min at 37 °C with horseradish peroxidase-conjugated goat antimouse IgG (diluted 1 : 2500; Sigma) in HBSS/PBS containing 5% FCS. Cells were then washed three times with 0.5-mL HBSS/PBS and incubated for 60 min at 37 °C in the dark with 0.25-mL, 0.003% H_2O_2 plus 0.1-mg/mL 3,3′, 5,5′-tetramethylbenzidine as a substrate. The reaction was stopped by adding 75 μL of 8 N H_2SO_4, and the samples were transferred to 96-well plates that were read at 450 nm on a plate reader. Control was performed by staining monolayers only with secondary antibody.

Treatment protocols

Before transmigration or surface expression assays, HUVEC monolayers were preincubated for 3 h in the presence or absence of the following K_{ATP} channel blockers: (1) gliclazide (20 μM), (Les Laboratories Servier, Neuilly sur Seine, France); (2) glibenclamide (10 μM, Sigma, St. Louis, MO); (3) glimepiride (5 μM), (Aventis Pharma, Tokyo, Japan); and (4) nateglinide (100 μM Ajinomoto, Tokyo, Japan). These doses were selected to match the maximum concentrations of these agents in human blood (C_{max}). We also employed 1-μM anisomycin (Calbiochem, Darmstadt, Germany) to activate MAP kinase. The optimum concentration of anisomycin was determined by preliminary dose- and time-dependent measurements of neutrophil transmigration across HUVEC and endothelial PECAM-1 expression (data not shown). To examine the relationship between the effects of antidiabetic agent(s) to NO metabolism, we used two NO synthase (NOS) inhibitors, N^G-nitro-L-arginine methyl ester hydrochloride (L-NAME; 30 μM) (Dojindo Co., Kumamoto, Japan) and N^5-(1-iminoethyl)-L-ornithine dihydrochloride (L-NIO; 3 μM, Dojindo Co., Kumamoto, Japan). The concentrations of NOS inhibitors used were determined from their IC_{50} values.

Cytotoxicity (LDH) assay

Cytotoxicity was measured using the TOX-7 LDH assay kit according to manufacturer's instructions (Sigma).

Apoptosis analysis

Apoptosis was measured by staining fixed monolayers with the Hoechst-33258 (Wako, Osaka, Japan) and examining nuclei for evidence of fragmentary nuclei.

Statistical analysis

All experiments were performed in triplicate with four different endothelial cell preparations. All values are expressed as means ± SEM. Data were analyzed using a one-way ANOVA with Fisher for multiple comparisons. A p-value < 0.05 was considered significant.

Results

Effects of K_{ATP} channel blockers on PMN migration across HUVEC and endothelial PECAM-1 expression induced by high insulin

Incubation of HUVEC with 100-μU/mL (0.609 nM) insulin for 24 h significantly increased PMN migration (21.7 ± 0.5%), as previously reported [15] (Figure 1). We confirmed that the effect of high insulin was not due to cytotoxicity or apoptosis, since there was no significant difference in cytotoxicity or apoptosis observed between untreated and high insulin–treated endothelial cells, using lactate dehydrogenase (cytotoxicity) or nuclear fragmentation (apoptosis) using Hoechst-33258 staining (data not shown). We next examined the effects of K_{ATP} channel blockers on PMN migration across HUVEC enhanced by high insulin, and found that the effect mediated by high insulin was attenuated significantly by gliclazide (20 μM), but not by glibenclamide (10 μM), glimepiride (5 μM), or nateglinide (100 μM) (Figure 1). In addition, we tested several concentrations of K_{ATP} channel blockers (glibenclamide, glimepiride or nateglinide) at doses equal to or exceeding the maximum concentrations of the agents found in human blood, and at several treatment times (3, 6, 12, 24 h). These agents all failed to inhibit high insulin–enhanced PMN migration (data not shown). On the other hand, different concentrations of gliclazide blocked high insulin–enhanced PMN migration. Compared to the control that showed 12.8 ± 0.3% migration, high insulin itself increased migration in this model to 21.7 ± 0.5% (Figure 1). Gliclazide treatment reduced the high insulin–induced migration. The 10-μM gliclazide, high insulin–treated groups showed 15.2 ± 0.5% migration, statistically different ($p > 0.001$) from high insulin alone. 20-μM gliclazide + high insulin dropped migration to 13.3 ± 0.4%, and this level was also significantly different ($p > 0.001$) from high insulin + 10-μM gliclazide. Lastly, 100-μM gliclazide + high insulin dropped migration to 12.6 ± 0.2%, and was again significantly different ($p > 0.01$) from high insulin + 20-μM gliclazide. However, the inhibitory effect of gliclazide was not affected by treatment times (3, 6, 12, 24 h) (data not shown). Gliclazide by itself did not affect baseline PMN chemotaxis (data not shown).

In addition, incubation of HUVEC with 100-μU/mL insulin for 24 h significantly enhanced endothelial PECAM-1 expression (0.403 ± 0.010), as previously reported [15] (Figure 2). Among the K_{ATP} channel blockers tested, only 20-μM gliclazide significantly attenuated insulin-induced PECAM-1 expression (Figure 2). Similarly, glibenclamide, glimepiride, and nateglinide, all failed to inhibit high insulin–enhanced endothelial

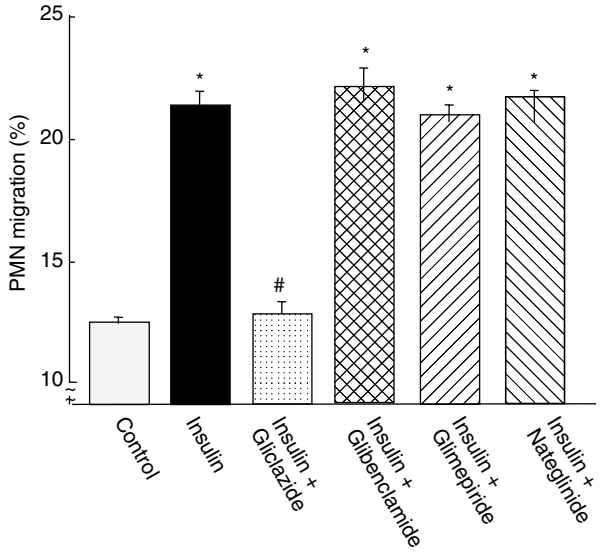

Figure 1. Effects of K_{ATP} channel blockers on PMN migration across HUVEC induced by high insulin. Cells were treated without (control) or with 100-μU/mL insulin for 24 h (insulin) in the presence or absence of 20-μM gliclazide, 10-μM glibenclamide, 5-μM glimepiride, and 100-μM nateglinide. Values are expressed as means ± SEM. *$p < 0.001$ compared to control, #$p < 0.001$ compared to the cells treated with high insulin alone

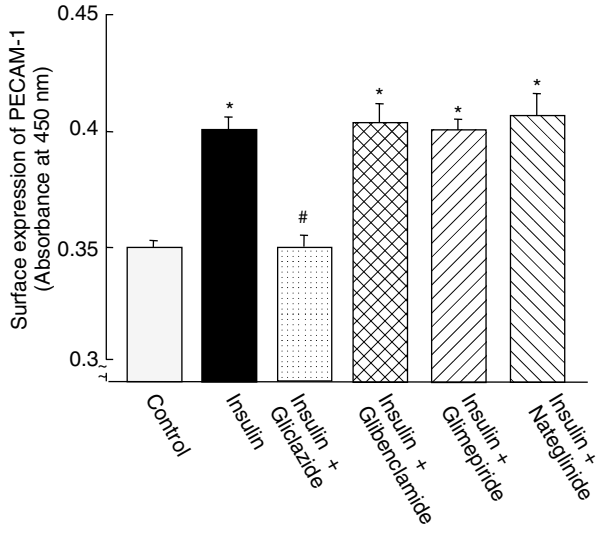

Figure 2. Effects of K_{ATP} channel blockers on endothelial PECAM-1 expression induced by high insulin. Cells were treated without (control) or with 100-μU/mL insulin for 24 h (insulin) in the presence or absence of 20-μM gliclazide, 10-μM glibenclamide, 5-μM glimepiride, and 100-μM nateglinide. Values are expressed as means ± SEM. *$p < 0.001$ compared to control, #$p < 0.001$ compared to the cells treated with high insulin alone

PECAM-1 expression at multiple doses (data not shown). However, gliclazide did attenuate high insulin–enhanced endothelial PECAM-1 expression at multiple doses (control: 0.349 ± 0.005; high insulin alone: 0.403 ± 0.010; high insulin + 10-μM gliclazide: 0.357 ± 0.004; high insulin + 20-μM gliclazide: 0.352 ± 0.003; high insulin + 100-μM gliclazide: 0.350 ± 0.002). The inhibitory effect of gliclazide was also not affected by the treatment times (3, 6, 12, 24 h) (data not shown). Gliclazide by itself did not affect basal endothelial PECAM-1 expression.

Role of MAP kinase in the gliclazide inhibition of PMN migration and endothelial PECAM-1 expression induced by high insulin

We reported previously that high insulin enhances PMN migration and PECAM-1 expression via MAP kinase pathways [15]. Therefore, we hypothesized that the protective actions of gliclazide might also be mediated by inhibition of MAP kinases. To evaluate this, we determined whether gliclazide attenuates neutrophil-endothelial cell interactions induced by a MAP kinase activator. Incubation of HUVEC for 3 h with 1-μM anisomycin (a MAP kinase activator) significantly increased both PMN migration across HUVEC and endothelial PECAM-1 expression, both of which were attenuated significantly by 20-μM gliclazide (Figures 3 and 4). Anisomycin at 1 μM did not induce either cytotoxicity or apoptosis of HUVEC (determined by a LDH assay and the Hoechst-33258 staining technique (cytotoxicity <1%, apoptotic cells <2%)). These findings suggest that gliclazide may decrease the endothelial events induced by high insulin through inhibition of MAP kinase pathways.

Role of endogenous NO in the protective actions of gliclazide on PMN migration induced by high insulin

To see whether endothelial NO production is related to the beneficial effects of gliclazide, we tested the

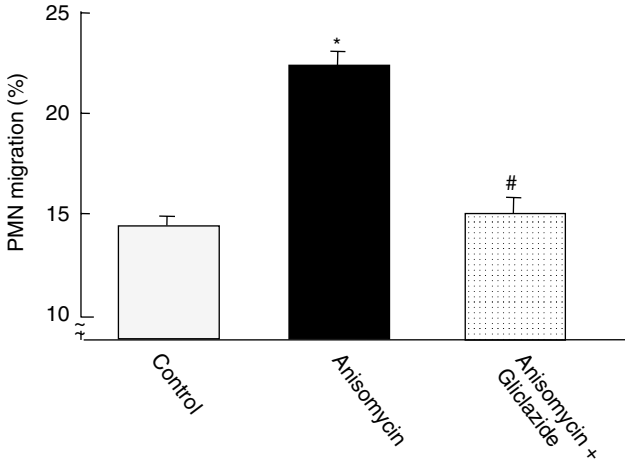

Figure 3. Participation of MAP kinase in the inhibitory action of gliclazide on PMN migration across HUVEC induced by high insulin. Cells were treated without (control) or with 1-μM anisomycin for 3 h (anisomycin) in the presence or absence of 20-μM gliclazide. Values are expressed as means ± SEM. *$p < 0.001$ compared to control, #$p < 0.001$ compared to the cells treated with anisomycin alone

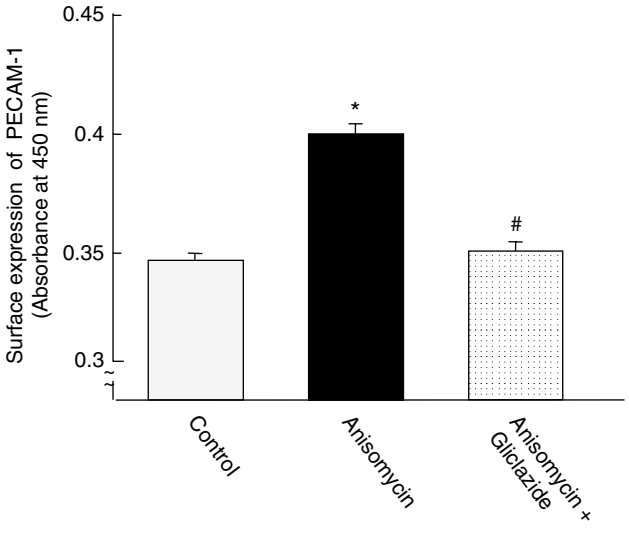

Figure 4. Participation of MAP kinase in the inhibitory actions of gliclazide on endothelial PECAM-1 expression induced by high insulin. Cells were treated without (control) or with 1-μM anisomycin for 3 h (anisomycin) in the presence or absence of 20-μM gliclazide. Values are expressed as means ± SEM. *$p < 0.001$ compared to control, #$p < 0.001$ compared to the cells treated with anisomycin alone

effects of the endothelial NOS inhibitor, L-NAME (30 μM), and of the inducible NOS inhibitor, L-NIO (3 μM), on gliclazide inhibition of PMN migration induced by insulin treatment. NOS inhibitors did not reverse the actions of gliclazide, suggesting that the endogenous NO production by endothelium is not involved in this event (Figure 5A and B). L-NAME and L-NIO by themselves also did not affect baseline PMN migration (data not shown).

Discussion

The insulin concentration used in this study, 100 μU/mL, is equivalent to the pathophysiological concentrations of insulin observed in insulin resistance, and blood concentrations exceeding 100 μU/mL can commonly be found in insulin-resistant individuals [20]. In this study, we evaluated the effects of various K_{ATP} channel blockers on insulin-enhanced neutrophil-transendothelial migration and endothelial PECAM-1 expression. We found that only gliclazide, but not other K_{ATP} channel blockers inhibited both events (Figures 1 and 2). The clinical relevance of these findings is particularly applicable because the gliclazide concentration used in the present study falls within the therapeutic range of 5 to 20 μM.

Several reports indicated that gliclazide-inhibited atherosclerotic lesions are induced by an atherogenic diet [21,22]. Gliclazide also prevents the progression of microvascular complications such as diabetic retinopathy and neuropathy, irrespective of the improvement of the glycemic control [23,24]. However, it is so far unknown whether gliclazide could modify the development of atherosclerotic diseases associated with hyperinsulinemia. Therefore, to the best of our knowledge, this study suggests the first evidence that at least gliclazide may provide an anti-inflammatory effect in the increased leukocyte-endothelial cell interactions seen during hyperinsulinemia, which also might contribute in preventing atherosclerotic disease. The inhibitory effect of gliclazide observed in this study is probably unrelated to K_{ATP} channels on endothelial cells, since, among K_{ATP} channel blockers, only gliclazide was effective in reversing high insulin–mediated neutrophil-endothelial cell interactions, despite the fact that gliclazide shares many common properties of typical K_{ATP} channel blockers (e.g. glibenclamide, glimepiride, and nateglurinide). Gliclazide is a member of K_{ATP} channel blockers that are available as oral antidiabetic agents, all of which produce their hypoglycemic action by blocking ATP-dependent potassium channels of pancreatic beta cells [25]. However, it has been reported that gliclazide also has a variety of nonmetabolic effects specifically involved in the vascular function aside from its effect on glycemic control, which other K_{ATP} channel blockers might not have [24,26–28].

For example, gliclazide was reported to restore impaired insulin signaling on insulin-resistant skeletal muscle cells by increasing tyrosine phosphorylation of the insulin receptor and increasing phosphatidylinositol (PI)-3 kinase activity [26]. It was also documented that gliclazide acts as a potent free-radical scavenger and inhibits tumor necrosis factor (TNF)-alpha production, platelet aggregation, and prostanoid release [24,27,28]. Most of these unusual effects may be mediated by the unique azabicyclo-octyl ring grafted on to its sulphonylurea core of gliclazide, which other K_{ATP} channel blockers do not share [21,27]. On the other hand, although K_{ATP} channels are present in a variety of cell types, it has been reported that among cell types, affinities of K_{ATP} channel blockers are largely different due to different types of sulphonylurea subunits of K_{ATP} channels [29,30]. Therefore, while unlikely, the different affinities of K_{ATP} channel blockers to endothelial cells could also account for the variation of inhibitory effects between gliclazide and other K_{ATP} channel blockers reported here.

Neutrophil-transendothelial migration is modulated by endothelial adhesion molecules, especially PECAM-1 [31]. The fact that PECAM-1 levels are increased in serum and cerebrospinal fluid within 24 h after the onset of stroke suggests the involvement of PECAM-1 in both inflammatory and atherosclerotic processes [32]. We reported that high insulin enhances neutrophil-transendothelial migration via increasing surface expression of PECAM-1 [15], and to obtain further insight into the mechanisms for the antimigratory effect of gliclazide in neutrophils, we investigated the effect of gliclazide on endothelial PECAM-1 expression. We observed that only gliclazide significantly attenuated the endothelial PECAM-1 expression enhanced by high insulin (Figure 2). Although little is known about the effect of gliclazide on PECAM-1 expression, it is possible that gliclazide inhibits the expression of other endothelial adhesion molecules, blocking other leukocyte-endothelial interactions. Gliclazide has

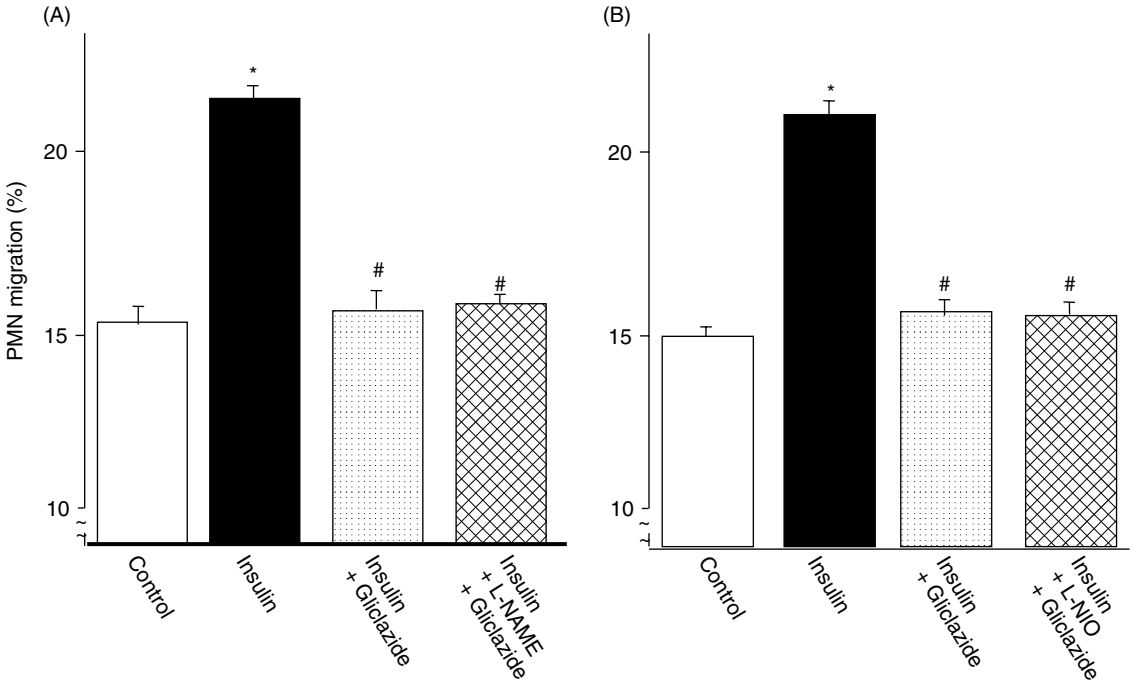

Figure 5. Effects of NOS inhibitors on the protective actions of gliclazide on the high insulin–induced PMN migration across HUVEC. Cells were treated without (control) or with 100-μU/mL insulin for 24 h (insulin) in the presence or absence of (A) an endothelial NOS inhibitor, 30-μM L-NAME, and (B) an inducible NOS inhibitor, 3-μM L-NIO. Values are expressed as means ± SEM. *$p < 0.001$ compared to the respective control, #$p < 0.001$ compared to the cells treated with high insulin alone

been shown to inhibit expression of intercellular adhesion molecule (ICAM)-1, vascular cell adhesion molecule (VCAM)-1, and E-selectin induced by glycated albumin in human endothelial cells [33]. Furthermore, we reported previously that gliclazide inhibited the expression of ICAM-1, E-selectin, and P-selectin in human endothelial cells induced by high glucose [34,35]. Therefore, the findings that protective activity of gliclazide against leukocyte-endothelial cell interactions through inhibiting the expression of various endothelial adhesion molecules may account for the inhibitory effect of gliclazide on endothelial PECAM-1 expression reported here.

We demonstrated previously that the actions of high insulin on the neutrophil-transendothelial migration and endothelial PECAM-1 expression were consistent with MAP kinase activation [15]. There is at least one more report demonstrating the association of PECAM-1 activation with a MAP kinase pathway [36]. Therefore, we evaluated the relationship between mitogen activated protein kinase (MAPK) and the inhibitory effects of gliclazide on the endothelial reactions to high insulin. We found that gliclazide attenuates MAP kinase activities because gliclazide blocked the effects of a MAP kinase activator, anisomycin, on both neutrophil-transendothelial migration and endothelial PECAM-1 expression, independent of insulin (Figures 3 and 4). Consistent with our data, recent studies suggested that gliclazide could improve an imbalance between insulin signaling of MAP kinase and phosphatidylinositol (PI) 3-kinase pathways in insulin-resistant skeletal muscle cells by restoring impaired PI 3-kinase activity [26].

While normal insulin maintains production of NO [37], one of the most important anti-inflammatory mediators, NO production is blunted in insulin resistance [16,17]. In addition, a previous report indicates that gliclazide improved the impaired vasodilatation, inadequately responsive to exogeneous NO in streptozotocin-induced diabetic rats [38]. Other studies demonstrate that an acetylcholine-induced exaggerated contraction of diabetic rabbit aorta in the presence of L-NAME was prevented by gliclazide treatment [25]. Therefore, using NOS inhibitors, we examined whether the protective effects of gliclazide are related to NO production by endothelial cells. Two different NOS inhibitors, L-NAME and L-NIO, did not modify the results, suggesting that endogenous NO does not appear to be involved in the protective effect of gliclazide (Figure 5A and B).

In conclusion, we demonstrated that gliclazide protects against high insulin–induced neutrophil-transendothelial migration and endothelial PECAM-1 expression through inhibition of MAP kinase activation, and are unrelated to NO metabolism. These findings may help to characterize the novel anti-inflammatory mechanisms of gliclazide in neutrophil-endothelial cell interactions in hyperinsulinemia. These results show that among K_{ATP} channel blockers, only gliclazide improves neutrophil-transendothelial migration produced by hyperinsulinemia. The appropriate use of gliclazide may eventually help prevent the development of atherosclerosis seen in patients with diabetes, particularly those complicated by hyperinsulinemia.

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