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Effects of Bisphosphonates on Glucose Transport in a Conditionally Immortalized Rat Retinal Capillary Endothelial Cell Line (TR-iBRB Cells)

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Abstract

The objective of the present study was to elucidate the effect of bisphosphonates, anti-osteoporosis agents, on glucose uptake in retinal capillary endothelial cells under normal and high glucose conditions. The change of glucose uptake by pre-treatment of bisphosphonates at the inner blood-retinal barrier (iBRB) was determined by measuring cellular uptake of [^{3}H]3-O-methyl glucose (3-OMG) using a conditionally immortalized rat retinal capillary endothelial cell line (TR-iBRB cells) under normal and high glucose conditions. [^{3}H]3-OMG uptake was inhibited by simultaneous treatment of unlabeled D-glucose and 3-OMG as well as glucose transport inhibitor, cytochalasin B. On the other hand, simultaneous treatment of alendronate or pamidronate had no significant inhibitory effect on [^{3}H]3-OMG uptake by TR-iBRB cells. Under high glucose condition of TR-iBRB cells, [^{3}H]3-OMG uptake was increased at 48 h. However, [^{3}H]3-OMG uptake was decreased significantly by pre-treatment of alendronate or pamidronate compared with the values for normal and high glucose conditions. Moreover, geranylgeraniol (GGOH), a mevalonate pathway intermediate, increased the uptake of [^{3}H]3-OMG reduced by bisphosphonates pre-treatment. But, pre-treatment of histamine did not show significant inhibition of [^{3}H]3-OMG uptake. The glucose uptake may be down regulated by inhibiting the mevalonate pathway with pre-treatment of bisphosphonates in TR-iBRB cells at high glucose condition.

Key Words: Glucose uptake, Bisphosphonates, Inner blood-retinal barrier, Retinal capillary endothelial cells, Mevalonate pathway

INTRODUCTION

Osteoporosis is a metabolic bone disease characterized by progressive decline in bone mass and bone quality, and increased risk of fracture. Diabetes, a chronic metabolic disorder, is also a major health problem and its prevalence is increasing rapidly. Recent meta-analyses and cohort studies have shown that type 1 and 2 diabetes are associated with higher fracture risk (Kim, 2013). Actually, therapeutics for osteophorosis such as bisphosphonates are frequently used for osteoporosis treatment in patients with diabetes mellitus. Bisphosphonates are the most commonly prescribed medication used to treat osteoporosis. They are also useful in hypercalcemia of malignancy, osteolytic bone metastasis and Paget disease of bone (Peterson and Bedrossian, 2012). In addition, recent reports have suggested that bisphosphonates showed anti-angiogenic effects via inhibition of production of the pro-angiogenic matrix metalloproteinase (MMP)-9 and vascular endothelial growth factor (VEGF) (Nagai *et al*., 2007). A clinical study has demonstrated a therapeutic effect of bisphosphonates in patients with neovascular age-related macular degeneration (AMD) (Honda *et al*., 2010). Furthermore, it was reported that bisphosphonates might be promising remedy for diabetic retinopathy through inhibition of the advanced glycation end products (AGEs), suppression of reactive oxygen species (ROS) and VEGF (Yamagishi *et al*., 2006; Yokota *et al*., 2007). Therefore, bisphosphonates may be an effective treatment modality for ocular neovascularization such as diabetic retinopathy.

Diabetic retinopathy is a major complication of diabetes mellitus and leads visual impairment and blindness. Previous report suggests that hyperglycemia initiates development of diabetic retinopathy (Engerman and Kern, 1984). Diabetic retinopathy is developed by increase of AGEs formation, oxidative stress, and aldose reductase activity and activation of protein kinase C (PKC) (Brownlee, 2001). It is based on glucose

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accumulation in retina. Retina constitutes a blood-retinal barrier (BRB) to supply nutrients and prevent nonspecific transport between the circulating blood and neural retina (Stewart and Tuor, 1994; Cunha-Vaz *et al*., 1996). BRB is composed of retinal capillary endothelial cells (iBRB) and retinal pigment epithelial cells (RPE, oBRB). The glucose supply to the retina from the circulating blood is mediated by a facilitative transporter, GLUT1 in iBRB and oBRB (Takata *et al*., 1992; Hosoya *et al*., 2001). Thus, the study of glucose uptake through BRB is essential for prevention of diabetic retinopathy. However, the regulation of glucose uptake at the BRB in response to bisphosphonates has not been elucidated.

In this study, we investigated the effect of bisphosphonates on glucose uptake on inner blood-retinal barrier cells cultured in normal or elevated glucose concentration. Glucose uptake at the iBRB was examined in a conditionally immortalized rat retinal capillary endothelial cell line (TR-iBRB cells), which maintains certain in vivo functions and is a suitable in vitro model for the iBRB (Hosoya *et al*., 2001). Our findings may contribute that bisphosphonates have the beneficial effects on the prevention of diabetic retinopathy in terms of glucose regulation in retina.

MATERIALS AND METHODS

Materials

[^{3}H]3-O-methyl glucose ([^{3}H]3-OMG) (1 mCi/mmol) was purchased from GE Healthcare (Chalfont St. Giles, UK). Alendronate, geranylgeraniol (GGOH), 3-OMG, taurine and histamine were purchased from Sigma Chemical (St. Louis, MO, USA). Pamidronate, D-glucose and L-ascorbic acid were purchased from TCI Co., Merck Co. and Junsei Chemical Co., respectively. All other chemicals were commercial products of reagent grade.

Cell culture

The TR-iBRB cells were cultured according to the previous report (Kang *et al*., 2009; Lee and Kang, 2013). TR-iBRB cells were grown routinely in rat tail collagen type 1-coated tissue culture dishes (Iwaki, Tokyo, Japan) at 33°C and cultured in a humidified atmosphere of 5% CO_2/air. The cells were cultured in Dulbecco's modified Eagle's medium (DMEM; WelGENE, Seoul, Korea) containing 5.5 mM glucose (normal) supplemented with 10% fetal bovine serum (WelGENE, Seoul, Korea), 15 μg/mL endothelial cell growth factor (Roche, Mannheim, Germany), 100 U/mL penicillin and 100 μg/mL streptomycin (WelGENE, Seoul, Korea).

[^{3}H]3-OMG uptake study in the TR-iBRB cells

TR-iBRB cells were cultured on rat tail collagen type 1-coated 24-well plates (Iwaki, Tokyo, Japan) at 33°C for 48 h. After removal of culture medium, cells were washed with 1 mL glucose-free extracellular fluid (ECF) buffer at 37°C. Uptake was initiated by addition of 200 μL glucose-free ECF buffer containing 1 μCi [^{3}H]3-OMG at 37°C in the absence or presence of inhibitors. The uptake was terminated at 30 s by removing the solution and washed with 1 mL ice-cold glucose-free ECF buffer. The cells were dissolved in 1 N NaOH overnight at room temperature and radioactivity was measured in a liquid scintillation counter (LS6500; Beckman, Fullerton, CA, USA). To investigate the effect of bisphosphonates on glucose transport under high glucose condition, the TR-iRBB cells were pretreated with 25 mM glucose for 48 h and the uptake study was performed as described above. TR-iBRB cells were pre-incubated with 100 nM to 10 μM of alendronate or pamidronate for 30 min. The concentrations were selected based on concentrations experienced by patients and those used in culture experiments. Especially, TR-iBRB cells were pre-treated with 10 μM of bisphosphonates either alone or in the presence of mevalonate pathway intermediates, 10 μM geranylgeraniol (GGOH).

Table 1. Effect of several compounds on [^{3}H]3-OMG uptake by TR-iBRB cells

Compounds	Concentration (mM)	% of control
Control		100 ± 5
D-glucose	30	32.5 ± 2.6**
3-OMG	30	65.1 ± 5.9*
Cytochalasin B	0.01	59.6 ± 4.2*
Alendronate	1	94.0 ± 5.1
Pamidronate	1	97.1 ± 3.6
Histamine	1	109 ± 3
L-ascorbic acid	1	104 ± 4
Taurine	1	110 ± 4

The [^{3}H]3-OMG uptake (1 μCi, 1.25 μM) was performed in the absence (control) or presence of several inhibitors at 37°C for 30 s. Each value represents the mean ± S.E.M. (n=3-4). *$p<0.01$, **$p<0.001$ significantly different from the control.

Data analysis

All data represent mean ± S.E.M. Statistical analyses were carried out by one-way ANOVA with Dunnett's post-hoc test and $p<0.05$ was considered statistically significant.

RESULTS

Inhibitory effect of several compounds on [^{3}H]3-OMG uptake by TR-iBRB cells

To confirm the existence of glucose transporter (GLUT1) function in TR-iBRB cells, we performed the inhibition study using substrates and inhibitors of GLUT1 in TR-iBRB cells. [^{3}H]3-OMG uptake was inhibited by D-glucose and unlabeled 3-OMG at 30 mM by 67.5% and 34.9%, respectively (Table 1). Moreover, glucose transport inhibitor, 10 μM cytochalasin B inhibited glucose uptake by more than 40% (Table 1). On the other hand, taurine at the concentration of 1 mM as a negative control did not have any significant effect (Table 1). This inhibition of [^{3}H]3-OMG uptake supports that facilitative glucose transporters are involved in the uptake process by TR-iBRB cells. In addition, glucose uptake was not inhibited by alendronate, pamidronate, histamine and L-ascorbic acid at the same concentration (Table 1).

Effect of bisphosphonates on the [^{3}H]3-OMG uptake in normal glucose condition by TR-iBRB cells

We investigated the change of [^{3}H]3-OMG uptake in TR-iBRB cells by bisphosphonates. Glucose uptake was significantly reduced by pre-treatment of bisphosphonates for 30 min. When the cells were pre-treated with various concentra-

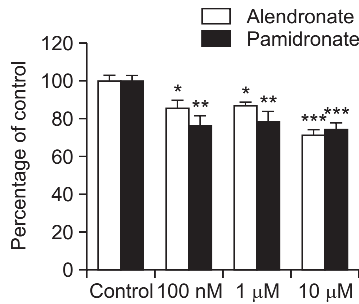

Fig. 1. Pre-incubation concentration dependency of bisphosphonates on [^{3}H]3-OMG uptake by TR-iBRB cells in normal glucose (5.5 mM) condition. TR-iBRB cells were pre-treated with 100 nM-10 μM alendronate (open) or pamidronate (closed) for 30 min. [^{3}H]3-OMG uptake was performed with glucose-free ECF buffer containing [^{3}H]3-OMG at 37°C for 30 s. Each point represents the mean ± S.E.M. (n=3-14). *p<0.05, **p<0.01, ***p<0.001, significantly different from normal glucose control.

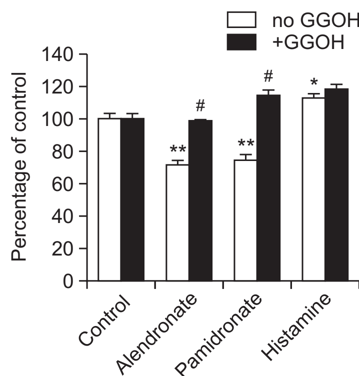

Fig. 2. Effect of GGOH on inhibition of glucose uptake by several compounds in normal glucose condition. TR-iBRB cells were pre-treated with 10 μM bisphosphonates or histamine in the absence (open) or presence of 10 μM GGOH (closed) for 30 min. [^{3}H]3-OMG uptake was performed with glucose-free ECF buffer containing [^{3}H]3-OMG at 37°C for 30 s. Results are means ± S.E.M. (n=4-14). *p<0.01, **p<0.001 significantly different from normal glucose control and $^{\#}p$<0.001, significantly different from (-)GGOH.

tions (100 nM, 1 μM or 10 μM) of alendronate or pamidronate for 30 min, glucose uptake was decreased in dose-dependent manner under normal glucose condition (Fig. 1).

Regulation of glucose transport by GGOH in normal glucose condition by TR-iBRB cells

To investigate the mechanism of glucose transport reduced by bisphosphonates, TR-iBRB cells were pre-incubated by bisphosphonates with GGOH for 30 min. As a results, GGOH restored [^{3}H]3-OMG uptake reduced by bisphosphonates in normal glucose condition (Fig. 2). In addition, TR-iBRB cells were pre-treated with 10 μM histamine which has angiogenic properties and is a little relevant with mevalonate pathway. Pre-incubation of histamine increased glucose uptake in normal glucose condition. GGOH had no difference compared with elevated glucose uptake by histamine (Fig. 2).

Table 2. Effect of high glucose (25 mM) on glucose transport in TR-iBRB cells

Glucose (mM)	% of control
5.5	100 ± 2
25	121 ± 4*

After TR-iBRB cells were pre-incubated in 5.5 mM or 25 mM glucose for 48 h, the [^{3}H]3-OMG uptake was performed at 37°C for 30 s. Each value represents the mean ± S.E.M. (n=10-15). *p< 0.001 significantly different from 5.5 mM glucose.

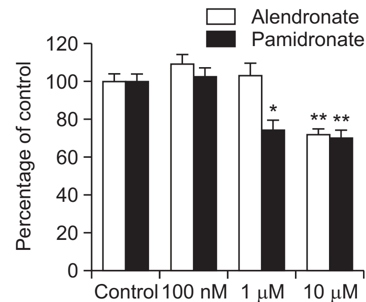

Fig. 3. Pre-incubation concentration dependency of bisphosphonates on [^{3}H]3-OMG uptake by TR-iBRB cells in high glucose (25 mM) condition. TR-iBRB cells were cultured in 25 mM glucose for 48 h. The cells were pre-treated with 100 nM-10 μM alendronate (open) or pamdronate (closed) for 30 min before uptake experiment. [^{3}H]3-OMG uptake was performed with glucose-free ECF buffer containing [^{3}H]3-OMG at 37°C for 30 s. Data were calculated as the mean ± S.E.M. (n=3-9). *p<0.01, **p<0.001, sig-nificantly different from high glucose control.

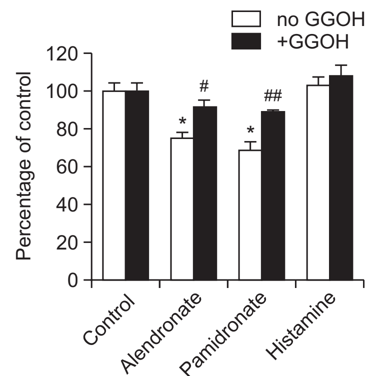

Fig. 4. Effect of GGOH on inhibition of glucose uptake by bisphosphonates and histamine in high glucose condition. TR-iBRB cells were cultured in 25 mM glucose for 48 h. The cells were pre-treated with 10 μM alendronate, pamidronate or histamine in the absence (open) or presence (closed) of 10 μM GGOH for 30 min before uptake experiment. [^{3}H]3-OMG uptake was performed with glucose-free ECF buffer containing [^{3}H]3-OMG at 37°C for 30 s. Each point represents the mean ± S.E.M. (n=3-9). *p<0.001, significantly different from high glucose control and $^{\#}p$<0.05, $^{\#\#}p$<0.01, significantly different from (-)GGOH.

Effect of bisphosphonates on the [^{3}H]3-OMG uptake by TR-iBRB cells in high glucose condition

TR-iBRB cells were cultured in 5.5 or 25 mM glucose for 48 h to investigate the inhibitory effect of bisphosphonates on

the glucose uptake in high glucose condition. The cells were pre-treated with bisphosphonates at concentration from 100 nM to 10 μM before [^{3}H]3-OMG uptake experiment. At high glucose condition, the glucose uptake was increased by a 1.2-fold (Table 2) and alendronate significantly inhibited glucose uptake at 10 μM. Pre-incubation of pamidronate at both 1 and 10 μM significantly decreased glucose uptake in high glucose condition (Fig. 3).

Restoration of glucose uptake by GGOH in TR-iBRB cells in high glucose condition

To investigate the mechanism of glucose uptake reduced by bisphosphonates in high glucose condition, TR-iBRB cells were pre-incubated by bisphosphonates with GGOH for 30 min at high glucose condition. The suppression of glucose uptake by bisphosphonates was restored by GGOH under high glucose condition (Fig. 4). In addition, glucose uptake was not affected by pre-treatment of 10 μM histamine and GGOH (Fig. 4).

DISCUSSION

The present study demonstrated that glucose uptake is regulated by pre-incubation of bisphosphonates in TR-iBRB cells, used as an in vitro model of the iBRB. 3-O-methyl-D-glucose (3-OMG), non-metabolic glucose analogue, was used as a model substrate for characterization of the D-glucose transport system (Betz and Goldstein, 1980).

Substrates of GLUT1, such as D-glucose and 3-OMG, and an inhibitor, cytochalasin B significantly inhibited [^{3}H]3-OMG uptake by TR-iBRB cells (Table 1). This inhibition in TR-iBRB cells is correspond with a previous in vitro bovine retinal uptake study which showed that glucose uptake into the retina is inhibited by substrates and inhibitors of GLUT1 (Betz *et al*., 1983). In contrast, when added to cells simultaneously 1 mM bisphosphonates, such as alendronate and pamidronate, glucose uptake did not have any significant effect (Table 1). These results suggest that alendronate and pamidronate are not mediated by GLUT1 into the cells. 1 mM bisphosphonates were the maximum concentration, which did not damage cell viability (Evans and Oberbauer, 2009). Moreover, L-ascorbic acid did not significantly inhibit glucose uptake although the structure of L-ascorbic acid is similar with that of glucose. It was reported that L-ascorbic acid is mainly transported in a form of dehydroascorbic acid via GLUT1 (Hosoya *et al*., 2004).

To investigate the effect of bisphosphonates on glucose transport in TR-iBRB cells, the cells were pre-treated with 100 nM-10 μM alendronate or pamidronate (Fig. 1). These concentrations were chosen because those correlate with the pharmacokinetics of bisphosphonates in soft tissue and used in culture experiments (Stepensky *et al*., 2002; Spreafico *et al*., 2006).

Our results showed that high glucose condition caused 1.2-fold increase in glucose uptake in TR-iBRB cells (Table 2). Previous study was also reported high glucose induced 1.9- and 2.5-fold increases in glucose uptake in hRPE and retinal vascular endothelial cells (hRVE cells), respectively (Busik *et al*., 2002). By exposing TR-iBRB cells to 10 μM alendronate or pamidronate for 30 min, [^{3}H]3-OMG uptake was significantly decreased by 72% and 70%, respectively, in comparison with high glucose control (Fig. 3). These results suggest that inhibition of glucose accumulation within retinal cells probably contributes at least in part to the observed inhibition of diabetic retinopathy by bisphosphonates.

To identify the regulatory mechanism of glucose transport in response to bisphosphonates at the inner BRB, the cells were incubated with 10 μM of alendronate or pamidronate either alone or in the presence of mevalonate pathway intermediates, 10 μM geranylgeraniol (GGOH), which is metabolized to geranylgeranylpyrophosphate and is used to recover geranylgeranylation (Fig. 2). Nitrogen-containing bisphosphonates such as alendronate and pamidronate are particularly able to inhibit pyrophosphate synthase in the mevalonate pathway (Luckman *et al*., 1998). Consequently, bisphosphonates decreased synthesis of the metabolite, GGOH in the mevalonate pathway. Therefore, supply of GGOH has been indicated to rescue mevalonate pathway inhibiting by bisphosphonates (Benford *et al*., 1999; Töyräs *et al*., 2003; Evan and Oberbauer, 2009). In previous report, addition of a GGOH interacted with alendronate restored VEGF protein secretion (Evans and Oberbauer, 2009). In another report, GGOH was able to restore the deregulated mevalonate pathway and to antagonize the effects of the biochemical block, through their anti-inflammatory and anti-oxidant activities (Tricarico *et al*., 2014). In our results, pre-incubation by bisphosphonates with GGOH, GGOH restored [^{3}H]3-OMG uptake reduced by bisphosphonates under normal and high glucose conditions (Fig. 2, 4). These results proposed the inhibitory effect of glucose uptake by bisphosphonates is due to interruption of geranylgeranylated pathway. Moreover, to clarify geranylgeranylation is associated with regulation of glucose uptake by bisphosphonates, the cells were pre-treated with histamine, which has pro-angiogenic effect. Histamine enhanced glucose uptake under normal glucose condition like previous study (Fig. 2), reported histamine stimulated glucose transport through H1-receptors (Thomas *et al*., 1995; Laurier *et al*., 2002). In high glucose condition, glucose uptake was not changed by histamine (Fig. 4). Moreover, pre-treatment of histamine with GGOH had no effect on glucose uptake in contrast with case of bisphosphonates, it may be related to another pathway (Connolly, 1991). Histamine, which is not related to mevalonate pathway, promotes an array of responses in endothelium, including hyperpermeability, endothelial cell growth, and enhanced glucose transport. Therefore, our results may suggest the relation of mevalonate pathway as mechanism of glucose uptake regulation at BRB by bisphosphonates. We will try to investigate the relation of glucose transporter (GLUT1) and bisphosphonates under high glucose condition in future study.

In conclusion, nitrogen containing bisphosphonates, such as alendronate and pamidronate, reduced glucose uptake by inhibiting the mevalonate pathway in TR-iBRB cells. Bisphosphonates might be useful for the prevention of diabetic retinopathy, at least in part, through inhibition of glucose uptake to the retina.

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