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Gliclazide Inhibits Proliferation but Stimulates Differentiation of White and Brown Adipocytes

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Gliclazide, a second-generation sulfonylurea, has anti-oxidant properties as well as hypoglycemic activities. In the present study, we investigated whether gliclazide affected proliferation and/or differentiation of HW white and HB2 brown adipocyte cell lines. Gliclazide inhibited proliferation of HW and HB2 cells in the medium containing fetal calf serum or epidermal growth factor (EGF). Gliclazide inhibited phosphorylation of EGF receptor and of extracellular signal-regulated kinase (ERK) 1/2 stimulated by EGF. Gliclazide increased lipid accumulation and peroxisome proliferator-activated receptor γ (PPARγ) expression in the early stage of differentiation of adipocytes. A K_{ATP} channel activator, diazoxide, did not inhibit the increase of lipid accumulation by gliclazide. Furthermore, gliclazide inhibited the DNA-binding activity of PPARγ in mature adipocytes. On the other hand, glibenclamide, other sulfonylurea, did not show these effects. These results indicate gliclazide inhibits proliferation and stimulates differentiation of adipocytes *via* down-regulation of the EGFR signalling. Gliclazide may have preventive and therapeutic effects on obesity, as well as on type 2 diabetes.

Key words: adipocyte, EGF, gliclazide, PPARγ, proliferation and differentiation.

Abbreviations: DEX, dexamethasone; EGF, epidermal growth factor; ERK, extracellular signal-regulated kinase; FCS, fetal calf serum; IBMX, 3-isobuthyl-1-methylxanthine; PPAR, peroxisome proliferator-activated receptor; PS, penicillin/streptomycin sulfate; RXR, retinoid X receptor; SUR, sulfonylurea receptor; TNF-α, tumor necrosis factor-α.

Metabolic syndrome has been a growing medical and sociological problem of the industrialized world. Obesity is recognized as the central and causal component in this syndrome, promoting atherosclerosis, hypertension and type 2 diabetes mellitus (*1*). Recent advances in adipocyte biology established fat tissue not only serves as a means of energy storage in the form of triglycerides but also exerts secretory or endocrine gland functions, producing various secretory molecules, such as tumor necrosis factor α (TNF-α), adiponectin, angiotensinogen II and leptin, referred to as adipocytokines. Elevated TNF-α in obese individuals leads to insulin resistance and then type 2 diabetes (*2*, *3*).

There are two types of adipocytes, white and brown (*4*). The main function of white adipocytes is to store energy molecules, triglycerides, while brown adipocytes play a role in thermogenesis, using triglycerides. White and brown adipocytes are able to transdifferentiate into each other (*5*). Their respective amounts vary in relation to strain, age, gender and environmental and nutritional conditions. Adipocytes play an important role in fat and glucose metabolism. The numbers and sizes of white adipocytes are determined by the long-term balance between energy intake and expenditure, which is influenced by the interaction with genetic, environmental and psychosocial factors (*1*). Human newborns have considerable amount of brown adipocytes like murine, whereas brown adipocytes in human adults are reduced (*4*).

Gliclazide is a second-generation sulfonylurea and commonly used to treat type 2 diabetes. It reduces blood glucose level by augmenting insulin release from pancreatic β-cells. Besides its hypoglycemic effect, gliclazide has been shown to possess anti-oxidant properties such as inhibition of LDL oxidation and reduction of platelet reactivity. We reported that gliclazide protected pancreatic β-cells from damage by hydrogen peroxide, and that it also protected 3T3-L1 adipocytes against hydrogen peroxide-induced insulin resistance with restoration of insulin-stimulated glucose transporter 4 (GLUT4) translocation (*6*, *7*). Sulfonylureas, such as gliclazide and glibenclamide, bind to sulfonylurea

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receptors (SUR) on cell surfaces and then block ATP-dependent potassium (K_{ATP}) channels. There are three SUR channel subtypes: SUR1 (β-cell type), SUR2A (cardiac and skeletal muscle type) and SUR2B (smooth muscle type). Sulfonylureas have a high affinity for SUR1, but a low affinity for SUR2. SUR1 is expressed on white adipocytes but not on preadipocytes (*8*).

Proliferation of adipocytes is regulated by several growth factors, such as fibroblast growth factor (FGF)-1, -2 and -10, epidermal growth factor (EGF) and insulin-like growth factor (IGF)-1 (*9*, *10*). The EGF family and its receptors, ErbB family, play important roles in cell proliferation, differentiation and survival in a large number of normal and tumorigenic cell lines (*11*). In adipocytes, EGF has been described to have distinct and sometimes controversial effects on adipogenesis (*12–14*). EGF blocks maturation of preadipocytes into mature adipocytes both *in vivo* and *in vitro*, but enhances adipogenesis in the already differentiating adipocytes. EGF receptor (EGFR) levels have been previously described either to remain constant or to decrease throughout differentiation. ErbB2, another member of the EGFR family, is down-regulated during the differentiation of murine 3T3-L1 fibroblastic cell line. EGFR and ErbB2 are down-regulated by differentiation inducers, such as 3-isobuthyl-1-methylxanthine (IBMX) and dexamethasone (DEX), in 3T3-L1 adipocytes (*15*).

Several transcriptional factors have been identified as potential regulators of adipocyte differentiation. Peroxisome proliferator-activated receptor (PPAR) γ is a key molecule among these factors (*16–18*). PPARs are transcription factors belonging to the nuclear receptor gene superfamily, and have three members, PPARα, β and γ. PPARγ is abundant in adipose tissue where it triggers adipocyte differentiation and lipid storage by regulating the expression of genes critical for adipogenesis.

In this study, we investigated the unknown function of gliclazide on proliferation and differentiation of white and brown adipocytes. We found that gliclazide, not glibenclamide, inhibited proliferation and stimulated differentiation of adipocytes *via* suppressing EGFR signalling.

EXPERIMENTAL PROCEDURES

Materials—Gliclazide was obtained from Dainippon Sumitomo Pharma (Osaka, Japan). Glibenclamide was purchased from Sigma (St Louis, MO, USA). Antibodies against EGFR and ErbB2 were purchased from Upstate (Lake Placid, NY, USA), and antibodies against pERK, ERK and PPARγ were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Cell Culture—Immortalized white preadipocyte HW cells and brown preadipocyte HB2 cells were provided by Prof Masayuki Saito (Tenshi College, Sapporo, Japan) (*19*). HW cells and HB2 cells were maintained in Dulbecco's modified Eagle's medium (D-MEM) supplemented with 10% fetal calf serum (FCS), 100 U/ml penicillin and 100 μg/ml streptomycin sulfate (PS). When cells grew to confluence (Day 0), cells were re-fed induction medium (D-MEM/10% FCS/PS/0.5 mM IBMX/1 μM DEX), during the induction period. After a 2-day incubation period (Day 2), cells were re-fed maintenance medium (D-MEM/10% FCS/PS/10 μg/ml insulin/50 nM 3-3′-5-triiodo-L-thyrosine) for 5–7 days to differentiate into mature adipocytes.

Immunoprecipitation and Western Blotting Analyses—To synchronize the cells in a quiescent state, the cells were cultured in starvation medium containing 0.5% FCS for 16 h and stimulated with 10 ng/ml EGF in the presence of the reagents to be tested. Cells were harvested and lysed with lysis buffer (1% Triton X-100, 1 mM sodium pyrophosphate, 1 mM sodium ortho vanadate, 10 mM sodium fluoride, 5 mM EDTA, 0.1 mM aprotinin and 1 mM phenylmethylsulfonyl fluoride in 20 mM Tris–HCl, pH 8.0). For phosphorylation detection of EGFR and ErbB2, the lysates were immunoprecipitated with anti-phosphotyrosine antibody (PY-20, BD, Franklin Lakes, NJ, USA), and then the sample was analysed by Western blotting, as described previously (*20*). For detection of pERK, ERK and PPARγ, the lysates were analysed by Western blotting using anti-pERK, ERK or PPARγ antibody. The immunoreactive bands were detected using an ECL kit (GE Healthcare, Buckinghamshire, UK) followed by a Lumino Imaging Analyzer (TOYOBO, Osaka, Japan). The density of the bands was quantified with NIH Image software.

Cell Proliferation Assay—HW and HB2 cells were seeded onto collagen-coated 96-well microplates (2×10^3 cells/well). The plates were incubated at 37°C for 24 h, and then re-fed medium containing samples to be tested. After 2 days, cell viability was measured at 450 nm absorbance using WST-1 reagents (Roche, Mannheim, Germany).

Triglyceride Accumulation Assay—HW and HB2 cells were seeded onto collagen-coated, 6 cm dishes. Cells were re-fed induction medium in the presence of the reagents to be tested, such as gliclazide, glibenclamide and diazoxide. On Day 4 (HB2 cells) or Day 5 (HW cells) after induction, cells were harvested and lysed. The supernatants were collected and the amount of triglyceride was measured at an absorbance of 600 nm using the Triglyceride kit WAKO (Wako, Osaka, Japan).

PPARγ Transcription Factor Binding Assay—HW and HB2 cells were seeded onto collagen-coated, 6 cm dishes. After maturation (Day 7), gliclazide or glibenclamide was added to the cells. The cells were incubated for 24 h and then harvested. The cells were incubated in 20 mM HEPES (pH 7.5) containing 5 mM sodium fluoride, 10 μM sodium molybdate and 0.1 mM EDTA for 15 min on ice, and then added final 1% Nonidet P-40. The samples were centrifuged, and the pellets were re-suspended in ice-cold extraction buffer (420 mM NaCl, 1.5 mM $MgCl_2$, 0.5 mM DTT, 1 mM sodium pyrophosphate, 10 mM sodium ortho vanadate, 20 mM sodium fluoride, 0.1 mM EDTA, 0.1 mM aprotinin, 1 mM phenylmethylsulfonyl fluoride and 25% glycerol in 10 mM HEPES, pH 7.9). After incubation for 30 min for 4°C, the samples were centrifuged at 15 000 r.p.m. for 10 min at 4°C, and the supernatants were collected as the cell nuclear extracts. Their DNA-binding activities of PPARγ were measured at an absorbance of 450 nm using a PPARγ Transcription Factor Assay kit (Cayman

Chemical, Ann Arbor, MI, USA), according to the manufacturer's instructions.

Statistical Analysis—The results are presented as mean ± SD of triplicate measurements. Comparisons between two groups were performed using the Student's *t*-test, where $P < 0.05$ was considered statistically significant.

RESULTS

Inhibition of Adipocyte Proliferation by Gliclazide—Gliclazide is known to be a multi-functional reagent, thus, we examined whether gliclazide had an effect on adipocyte proliferation. We estimated that 100 μM gliclazide was pharmacologically equivalent to 3 μM glibenclamide, according to matched clinical dosage and plasma levels (*21*, *22*). White preadipocyte HW cells and brown HB2 cells were incubated in the medium containing 2% FCS with gliclazide or glibenclamide for 2 days, and then their proliferation activity was measured using WST-1 reagents. There was a linearity between cell numbers and absorbance within the range. Gliclazide slightly decreased growth of both HW and HB2 cells in a dose-dependent manner, while glibenclamide had no inhibitory effects (Fig. 1A).

As EGF was reported to regulate adipogenesis, we examined the effect of gliclazide on adipocytes stimulated by EGF. EGF increased proliferation of HW and HB2 cells, and gliclazide reduced the growth stimulation of preadipocytes (Fig. 1B and C). On the other hand, glibenclamide had no inhibitory effects.

Inhibition of EGFR Signalling Pathway by Gliclazide—As EGF stimulated the proliferation of HW and HB2 cells, we examined expression of EGFR and ErbB2. The amounts of EGFR expression were approximately constant through the differentiation in HW and HB2 cells. ErbB2 expression decreased in HW and HB2 cells as preadipocytes differentiated into mature adipocytes. In HB2 cells, the expression of ErbB2 was decreased remarkably after Day 4 (Fig. 2A).

Next, we investigated the effect of gliclazide on intracellular signalling stimulated by EGF. The amount of phosphorylation of EGFR was decreased on HW (Fig. 2B) and HB2 (data not shown) cells treated with gliclazide, but not with glibenclamide. We also analysed phosphorylation of ErbB2, but failed to detect it even under control conditions as ErbB2 was expressed at low levels in mature adipocytes (data not shown). Furthermore, we examined the phosphorylation of extracellular signal-regulated kinase 1 and 2 (ERK 1/2), which belong to a family of mitogen-activated protein kinase (MAPK). The amount of phosphorylation of ERK 1/2 was also decreased on HW (Fig. 2C) and HB2 (data not shown) cells treated with gliclazide, but not with glibenclamide, while the amount of ERK 1/2 expression was constant.

Stimulation of Adipocyte Differentiation by Gliclazide—Gliclazide had an inhibitory effect on the proliferation of preadipocytes. We examined whether gliclazide stimulated preadipocyte differentiation. We evaluated lipid accumulation in the cytoplasm of adipocytes by measuring triglyceride content. HW and HB2 cells were supplemented with differentiation inducers, gliclazide or glibenclamide, and then cultured for 4–5 days. In HW cells, gliclazide significantly increased lipid accumulation, but glibenclamide had no definite

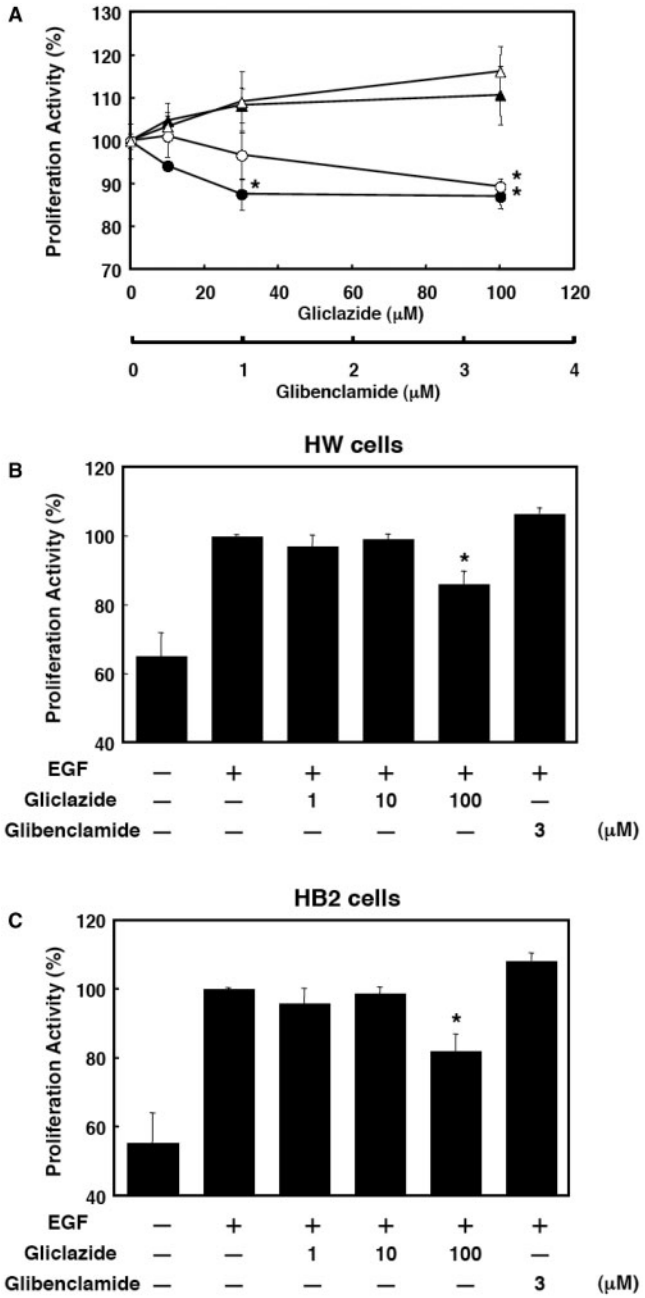

Fig. 1. **Inhibition of adipocyte proliferation by gliclazide.** (A) HW (open) and HB2 (closed) preadipocytes were re-fed medium containing 2% FCS in the presence of gliclazide (circles) or glibenclamide (triangles). After 2 days, the cells were incubated with WST-1 reagent for 1 h, and measured at 450 nm absorbance. $^*P < 0.05$ versus glibenclamide treatment. HW (B) and HB2 (C) preadipocytes were re-fed medium containing 0.5% FCS in the presence or absence of 10 ng/ml EGF, gliclazide or glibenclamide. After 24 h incubation, the cells were incubated with WST-1 reagent for 1 h and measured at 450 nm absorbance. $^*P < 0.05$ versus EGF treatment.

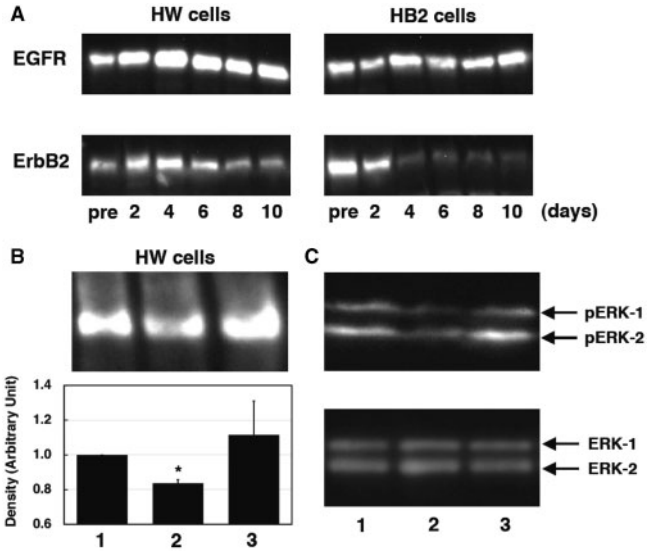

Fig. 2. **Changes in the intracellular molecules in adipocytes by glicalzide**. (A) HW and HB2 cells were harvested at 0, 2, 4, 6, 8 and 10 days after treatment with differentiation inducers. The cell lysates were analysed by Western blotting with anti-EGFR or ErbB2 antibodies. (B) Mature HW cells were re-fed medium containing 0.5% FCS in the presence of gliclazide or glibenclamide for 16 h. The cells were stimulated with EGF and then harvested. Phosphorylated EGFR was immunoprecipitated from whole cell lysate with anti-phosphotyrosine antibody and analysed by Western blotting with anti-EGFR antibody. (C) Whole cell lysates prepared as described earlier were analysed by Western blotting with anti-pERK antibody (upper panel) and anti-ERK antibody (lower panel). Lane 1, control; Lane 2, 100 μM gliclazide; Lane 3, 3 μM glibenclamide. $^{*}P < 0.05$ versus control treatment.

stimulation effect (Fig. 3A). In HB2 cells, 60 μM gliclazide only showed statistical differences compared to 2 μM glibenclamide (Fig. 3B). On the other hand, neither gliclazide nor glibenclamide had an effect on lipid accumulation compared to control treatments when individually added to mature HW and HB2 cells (Fig. 3C).

In order to examine whether gliclazide functioned through SUR, a K_{ATP} channel activator, diazoxide, was used. Diazoxide as well as gliclazide stimulated lipid accumulation in HW preadipocytes, and co-stimulation by gliclazide and diazoxide additionally increased the amount of lipid accumulation. Co-stimulation by diazoxide and glibenclamide, which had no stimulation effect, had the same level of lipid accumulation as that by diazoxide and gliclazide (Fig. 3D). This suggests gliclazide utilized a different pathway from the K_{ATP} channel, in order to accumulate triglycerides in the cytoplasm.

We also evaluated the expression of PPARγ as a marker of the early stage of differentiation in adipocytes. When gliclazide was added to the cells at the induction stage, the amount of PPARγ increased in a dose-dependent manner, but did not respond to glibenclamide (Fig. 4A). These results suggest gliclazide acted at the early period of differentiation and had no effects on lipid accumulation in mature adipocytes.

Inhibition of PPARγ Activity by Gliclazide—Finally, we examined the effect of gliclazide on the transcriptional activity of PPARγ. Gliclazide was added to mature adipocytes for 24 h, and then the DNA-binding activity of PPARγ was evaluated. The DNA-binding activity of PPARγ was decreased by gliclazide in a dose-dependent manner in both HW and HB2 cells, but not by glibenclamide (Fig. 4B and C).

DISCUSSION

Gliclazide, a second-generation sulfonylurea used in the treatment of type 2 diabetes mellitus, is a multifunctional reagent. Gliclazide was originally known to reduce blood glucose levels by augmenting insulin release from pancreatic islets. Besides its hypoglycemic effect, gliclazide is now known to function as a free radical scavenger (*6*, *7*). In this study, we investigated the unknown activity of gliclazide, reporting gliclazide inhibited proliferation of HW and HB2 preadipocytes but stimulated differentiation of these cells. Obesity is a major risk factor of type 2 diabetes. Adipogenesis is regulated by several growth factors, such as IGF-1, EGF and FGFs (*9*, *10*). It has been reported EGFR and ErbB2 are expressed in adipocytes *in vivo* and 3T3-L1 cell lines (*12–14*). When 3T3-L1 preadipocytes were re-fed medium containing the differentiation inducer, IBMX and DEX, the expressions of EGFR and ErbB2 were down-regulated (*15*). In HW and HB2 cells, the expression of EGFR was approximately constant, when preadipocytes were differentiated into mature adipocytes using IBMX and DEX (Fig. 2A). However, the expression of ErbB2 was decreased in the early stage of differentiation, especially in HB2 cells. The differences in expression pattern of ErbB2 might contribute to the characteristics of white and brown adipocytes.

EGF stimulated the proliferation of HW and HB2 cells, and gliclazide decreased that proliferation stimulated by EGF (Fig. 1B and C). EGFR and ErbB2 were expressed in HW and HB2 preadipocytes, but the expression of ErbB2 was decreased in mature HW and HB2 cells (Fig. 2A). Gliclazide inhibited phosphorylation of EGFR and its signal transduction molecule ERK 1/2 (Fig. 2B and C). This suggests that gliclazide regulated proliferation of adipocytes in part *via* the EGFR signalling. The mechanisms by which gliclazide inhibited phosphorylation of EGFR and ERK 1/2 remain unknown. It has been reported that PPARγ agonist ciglitazone increased phosphorylation of EGFR and ERK 1/2 *via* Src phosphorylation and antioxidant *N*-acetyl-L-cysteine attenuated the phosphorylation of these molecules (*23*). One possible mechanism is that gliclazide reduced Src activation by its antioxidant activity and decreased phosphorylation of EGFR and ERK 1/2. Other possibilities are that gliclazide affects the ligand binding to EGFR, or that it decrease the dimerization rate of EGFR.

Gliclazide induced differentiation of preadipocytes into mature adipocytes, as it induced lipid accumulation and PPARγ expression of adipocytes (Figs 3A and 4A). In HB2 cells, the differences between the two reagents were not so obvious, compared with HW cells (Fig. 3A and B), possibly for the reason that the differentiation

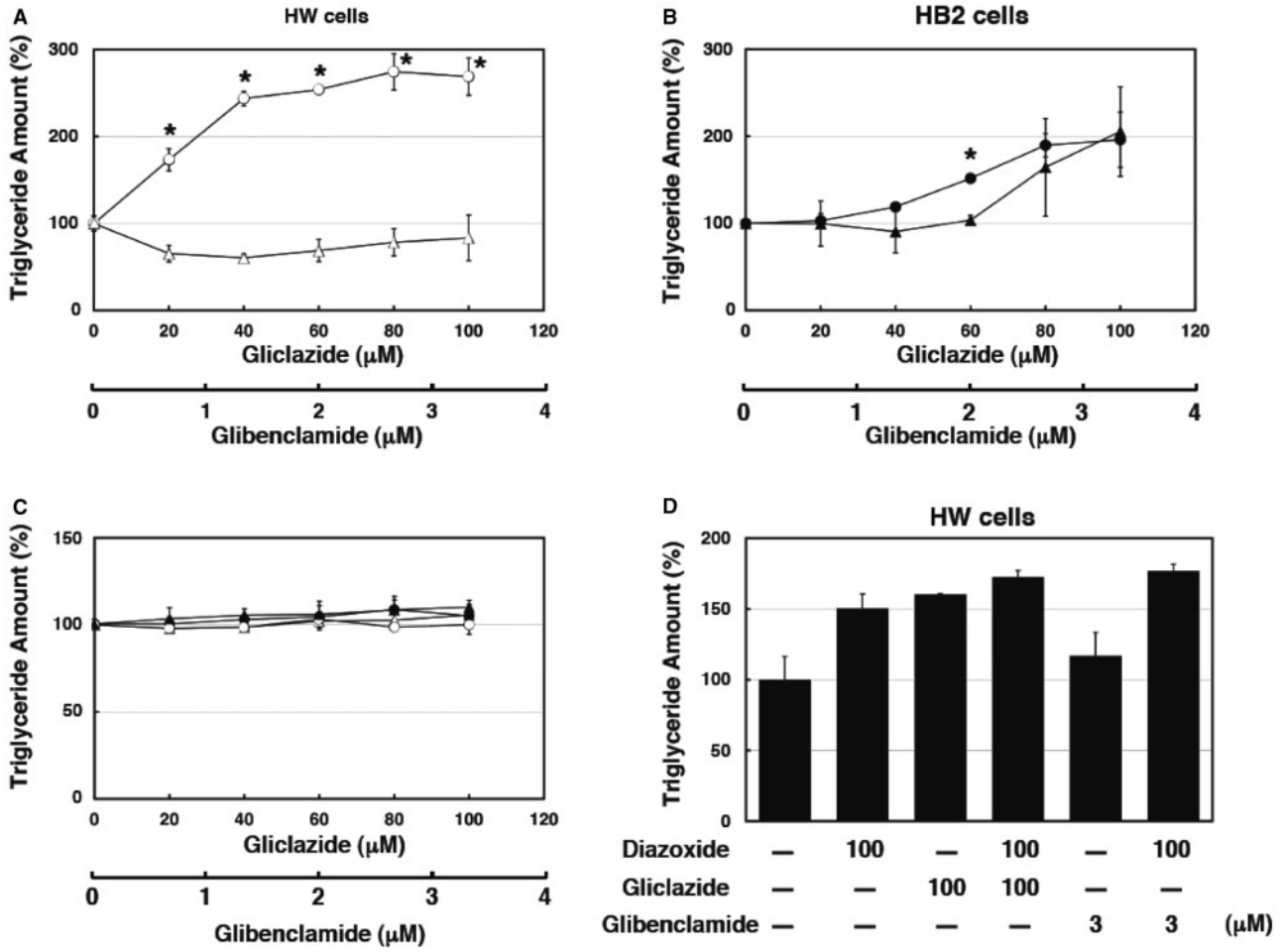

Fig. 3. **Lipid accumulation in adipocytes by gliclazide.** HW (A) and HB2 (B) preadipocytes were re-fed induction medium in the presence of gliclazide (circles) or glibenclamide (triangles). Cells were harvested at Day 4 (HB cells) or 5 (HW cells), and then the triglyceride amount in cell lysate was measured at 600 nm absorbance. $^{*}P < 0.05$ versus glibenclamide treatment. (C) Mature HW (open) and HB2 (closed) adipocytes were re-fed medium in the presence of gliclazide (circles) or glibenclamide (triangles). After 3 days, the cells were harvested and the triglyceride amounts in cell lysate were measured. (D) HW preadipocytes were re-fed induction medium in the presence of gliclazide, glibenclamide, or diazoxide. The cells were harvested at Day 5, and then the triglyceride amount in cell lysate was measured.

rate of HB2 cells was higher than that of HW cells (data not shown). We observed no effect of gliclazide on lipid accumulation in mature adipocytes (Fig. 3C). Therefore, gliclazide seems to stimulate the differentiation, but not the enlargement of adipocytes. These findings suggest gliclazide has preventive effects against obesity rather than therapeutic effects.

Gliclazide induced the expression of PPARγ in the early stage of differentiation (Fig. 4A), whereas it inhibited the transcriptional activity of PPARγ in mature adipocytes (Fig. 4B and C). These results seem somewhat contradictory, but PPARγ appears to play varying roles in differentiating and mature adipocytes. PPARγ is a ligand-dependent transcriptional regulator that heterodimerizes with retinoid X receptors (RXRs) (*18*). In preadipocytes, thiazolidinedione, an anti-diabetic agent, activates the PPARγ activity and stimulates differentiation into mature adipocytes (*24*, *25*). This is consistent with our findings that gliclazide stimulated PPARγ expression and lipid accumulation. On the other hand, it has been reported PPARγ requires the different RXR heterodimeric partner for lipogenic and vital functions in mature adipocytes, and that PPARγ-deficient mature adipocytes die within a few days (*18*). This suggests gliclazide reduces the survival of mature adipocytes, and has beneficial effects on obesity.

Both gliclazide and glibenclamide belong to sulfonylurea drugs and bind SUR. It was reported that SUR1 was expressed in human mature white adipocytes, but not the preadipocytes (*8*). Similarly, we observed that SUR1 and SUR2 were expressed in mature HW and HB2 cells, but not in preadipocytes (data not shown). Although sulfonylureas induce apoptosis in β-cells *via* SUR1 (*26*), inhibition of proliferation in HW and HB2 cells seemed not to be due to SUR1 signalling because of the absence of SUR1 in preadipocytes. Co-stimulation by gliclazide and diazoxide, a K_{ATP} channel activator, additionally increased the amount of lipid accumulation.

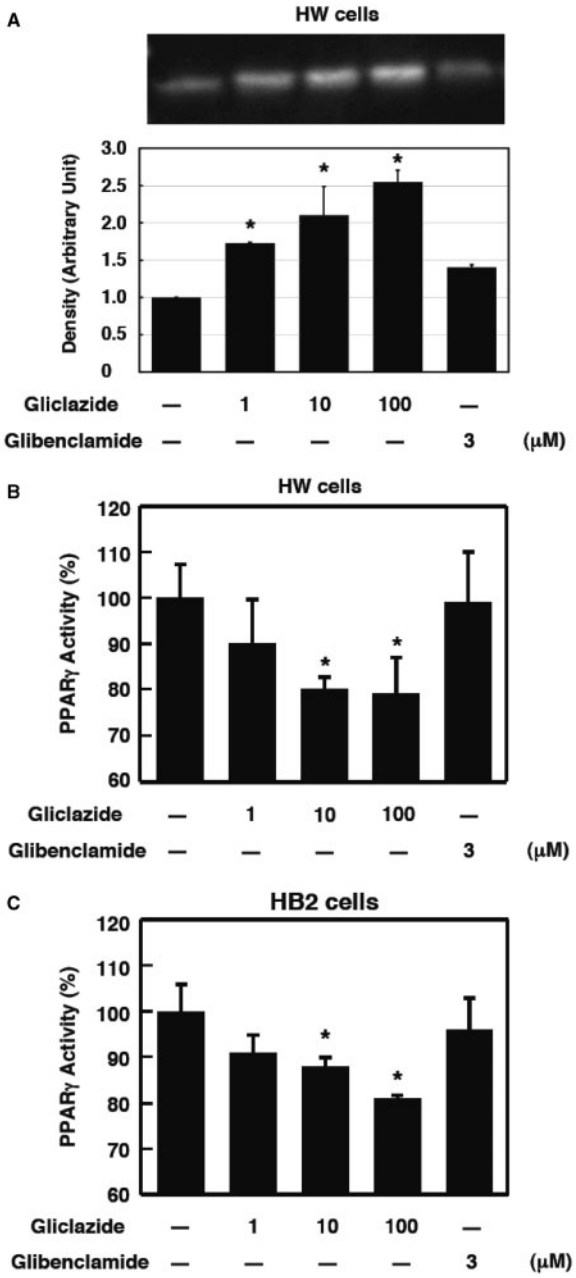

Fig. 4. **Effect of gliclazide on PPARγ activity.** (A) HW preadipocytes were re-fed induction medium in the presence of gliclazide or glibenclamide. After 5 days the cells were harvested. The lysates were analysed by Western blotting with anti-PPARγ antibody. Mature HW (B) and HB2 (C) adipocytes of Day 7 were re-fed medium in the presence of gliclazide or glibenclamide. After 24 h incubation, the cells were harvested. The nuclear extracts were collected, and then the DNA-binding activities of PPARγ were measured at 450 nm absorbance. $^{*}P < 0.05$ versus control treatment.

This suggests gliclazide had other molecular functions in adipocytes than that *via* SUR signalling. Gliclazide possesses a sulfonylurea moiety and an azabicyclo-octyl ring in its chemical structure. Both gliclazide and glibenclamide have the sulfonylurea moiety, which provides an insulinotropic property. The azabicyclo-octyl ring is thought to be responsible for the anti-oxidant property of gliclazide, although glibenclamide does not contain the azabicyclo-octyl ring (7). Further studies are necessary to elucidate the molecular mechanisms involved in the proliferation and differentiation of adipocytes by gliclazide.

This is the first study to demonstrate gliclazide inhibits proliferation, stimulates differentiation of adipocytes *via* down-regulation of the EGFR signalling, and also inhibits the PPARγ activity of mature adipocytes. Thus, gliclazide has the potential to inhibit the increase of preadipocytes and reduce the survival of mature adipocytes. Therefore, gliclazide may have preventive and therapeutic effects on obesity, as well as type 2 diabetes.

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