Antidiabetic Effect of a Prodrug of Cysteine, L-2-Oxothiazolidine-4-carboxylic Acid, through CD38 Dimerization and Internalization*

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CD38 is a bifunctional enzyme synthesizing (ADP-ribosyl cyclase) and degrading (cyclic ADP-ribose (cADPR) hydrolase) cADPR, a potent Ca\(^{2+}\) mobilizer from intracellular pools. CD38 internalization has been proposed as a mechanism by which the ectoenzyme produced intracellular cADPR, and thiol compounds have been shown to induce the internalization of CD38. Here, we show that the disulfide bond between Cys-119 and Cys-201 in CD38 may be involved in CD38 dimerization and internalization. We tested the effect of a reducing agent, L-2-oxothiazolidine-4-carboxylic acid (OTC), a prodrug of cysteine, on CD38 internalization in pancreatic islets. OTC enhanced insulin release from isolated islets as well as CD38 internalization and cytoplasmic Ca\(^{2+}\) level. Furthermore, islet cells treated with antisense CD38 oligonucleotide showed inhibition of OTC-induced insulin secretion. Intake of OTC in db/db mice ameliorated glucose tolerance, insulin secretion, and morphology of islets when compared with control mice. These data indicate that OTC improves glucose tolerance by enhancing insulin secretion via CD38/cADPR/Ca\(^{2+}\) signaling machinery. Thus, OTC may represent a novel class of antidiabetic drug.

CD38, a human lymphocyte antigen, is a bifunctional enzyme that catalyzes the synthesis (ADP-ribosyl cyclase) and hydrolysis (cyclic ADP-ribose (cADPR) hydrolase) of a cADPR (1, 2), and the latter is a potent Ca\(^{2+}\) mobilizer believed to be involved in Ca\(^{2+}\)-induced Ca\(^{2+}\) release via the ryanodine receptor/Ca\(^{2+}\) channel, thus serving as a second messenger in a variety of cells, such as pancreatic islet cells (3–9). ADP-ribosyl cyclase was first purified from the ovotestis of the Aplysia californica (10, 11). Later, the membrane-bound form, CD38, which has catalytic activities of not only ADP-ribosyl cyclase but also cADPR hydrolase, was cloned from different species (12–14). CD38s have highly conserved 12 cysteine residues in their extracellular domain. Aplysia ADP-ribosyl cyclases have homology of the 12 cysteine residues but two amino acid residues; cysteine 119 and cysteine 201 in the human CD38 are replaced by lysine and glutamic acid, respectively (15). When the cysteine 119 and cysteine 201 were replaced with lysine and glutamic acid, respectively, by site-directed mutagenesis, the CD38 mutant resulted in the disappearance of cADPR hydrolase activity but not cyclase activity (15). This finding demonstrates that cysteine 119 and cysteine 201 in CD38 play an important role in cADPR hydrolysis and also explains why Aplysia enzyme lacks cADPR hydrolase. An inference from the crystal structure of Aplysia cyclase is that CD38 has six disulfide bonds instead of the five bonds of the Aplysia enzyme. The sixth disulfide bond connecting the cysteine 119 and cysteine 201 of CD38 may be important for cross-linking to other molecules, and their reduction may render the hinge region less flexible (16).

EXPERIMENTAL PROCEDURES

Materials—L-2-oxothiazolidine-4-carboxylic acid (OTC) was obtained from Sigma and freshly prepared by dissolving the chemical in phosphate-buffered saline (PBS) and adjusting the pH to 7.2 with 3 N NaOH.

Site-directed Mutagenesis—Site-directed mutagenesis of CD38 was performed using the Mutan-K™ mutagenesis kit (Takara, Kyoto, Japan). The following oligonucleotides were used for the mutagenesis: 5'-AATCTTGTGTTAGTGCACGTC-3' (C119R), 5'-GCCACATCT-TCGGCAGCTTCT-3' (C201E), for human CD38, where underlined nucleotides were altered. The right mutant clones were selected by sequencing. A plasmid CMV vector carrying either the wild type or mutant human CD38 cDNA was introduced and expressed in HeLa cells.

Construction of Green Fluorescent Protein (GFP)-CD38—The expression construct encoding CD38-enhanced green fluorescent protein (GFP) was constituted by the following. The coding sequence of CD38 was PCR-amplified from pFLAG-CD38 (CD38 cDNA in pFLAG-CMV vector) using the sense primer, 5'-GGAAAGCTTGGCAAACTGGCAG-3' (the HindIII site is underlined, the start codon of CD38 is bolded), and the antisense primer, 5'-GGTACCTGCGAGAATTCGACG-3' (the SalI site is underlined). Specially, the CD38 stop codon of the antisense primer was changed from the nucleotides (TAA), which code for the translation stop, to the nucleotides TTG, which code for the amino acid leucine. The PCR products were cloned into pCMV-hGH using the Expand Cloning Kit and subcloned into the TOPO TA cloning kit (Invitrogen). Then, after restriction digestion with HindIII and SalI, the PCR product was ligated with identically prepared pEGFP-N1 vector (CLONTech) to create pEGFP-CD38. The expression construct encoding the fusion protein between EGFP and the CD38 variant from pFLAG-CMV vector and Jurket CD38 were constructed by a whole plasmid amplification strategy.

Functional Expression of CD38—A plasmid CMV vector carrying human CD38 cDNA or GFP-human CD38 cDNA was purified and linearized by digestion with PvuI. HeLa cells (10⁶ cells/ml) were transfected with the linearized DNA (10 µg) by LipofectAMINE transfection reagents.
(Roche Molecular Biochemicals). Stable transfectants were selected for resistance to G418 (200 μg/ml), and positive clones were repetitively selected with anti-CD38 antibody-coated Immulon beads.

**Enzyme Activities**—ADP-ribosyl cyclase and cADPR hydrolase assays were performed as described elsewhere (17) with HeLa cell homogenates containing equal amounts of enzyme proteins of wild type and mutant, as estimated by Western blot analysis.

**cADPR Measurement**—HeLa cells (10⁶ cells/ml) were incubated with anti-CD38 antibody (HB7, 1 μg/ml) for 30 min in ice and washed with Hank’s balanced salt solution (HBSS) (2 mM CaCl₂, 145 mM NaCl, 5 mM KCl, 1 mM MgCl₂, 5 mM d-glucose, 20 mM Hepes (pH 7.3)). CD38 ligation was performed by incubation of cells with anti-mouse IgG Fab’₂ for 3 min at 37°C. After washing with ice-cold PBS, the cells were immediately frozen in liquid nitrogen and stored at −80°C. The frozen cells were added to 8% trichloroacetic acid to extract cADPR. The cADPR levels were measured by a specific radioimmunoassay described elsewhere (18).

**Intracellular Ca²⁺ Measurement in HeLa Cells**—HeLa cells were incubated with Fura-2/AM (4 μM) in RPMI 1640 medium containing 3% fetal bovine serum for 60 min at 37°C. The Fura-2/AM-loaded cells were incubated with anti-CD38 antibody (HB7, 1 μg/ml) for 30 min in ice and washed with HBSS. For the fluorometric measurement of Ca²⁺, 1 × 10⁶ cells were placed in a quartz cuvette in a thermostatically controlled cell holder at 37°C, and the cell suspension was stirred continuously. After the addition of rabbit anti-mouse IgG Fab’₂, fluorescence ratios were taken with an alternative wavelength time scanning method (dual excitation at 340 and 380 nm; emission at 510 nm) using a spectrofluorometer (Photon Technology International, Princeton, NJ).

**Preparation of Islets**—Pancreatic islets were isolated from male BALB/c mice weighing 20–25 g. Animals were fed ad libitum or fasted overnight before isolation of islets. Islets were isolated according to the collagenase method (19) with some modification. Pancreas of ether-anesthetized mice was distended by infusion of HBSS (pH 7.3) containing 1.5 mg/ml Type V collagenase (Sigma) via the common bile duct. After removing destroyed acinar cells by washing with HBSS, intact islets were handpicked with a Pasteur micropipette under a dissecting microscope.

**Insulin Assay in Pancreatic Islet Cells**—For the test of OTC dose dependence in insulin release, islets were incubated with Krebs-Ringer bicarbonate buffer (KRB), pH 7.4, containing 0.01% bovine serum albumin (BSA), 16 mM Hepes, and 2.8 mM glucose for 60 min at 37°C. After washing with the above buffer, the cells were further incubated with KRB containing 0.01% BSA, 16 mM Hepes, and 5.6 mM glucose in the presence of various doses of OTC for 60 min, and released insulin in the supernatant was measured by radioimmunoassay using an insulin assay kit (Amersham Biosciences, Inc., Buckinghamshire, UK). To evaluate the effect of OTC in the presence of different glucose concentrations, the cells were incubated with KRB containing 0.01% BSA, 16 mM Hepes, and 1 mM OTC in the presence of different glucose concentrations (2.8, 5.6, and 17.5 mM) for 60 min, and released insulin in the supernatant was measured.

**Intracellular Ca²⁺ Measurement in Islet Cells**—Islet cells were trypsinized and cultured on polylysine-coated cover glass (MatTek Corporation, Ashland, Massachusetts). The cells were incubated with Fluo-3 AM (20 μM) in KRB solution (pH 7.4) containing 2.8 mM glucose for 30 min at 37°C. After washing with KRB solution containing 2.8 mM glucose, the cells were incubated with or without 1 mM OTC for 5 min before the addition of 22.2 mM glucose. Fluo-3 fluorescence in the cells was excited at 488 nm from a high power argon laser, and emissions at wavelengths longer than 520 nm were detected by photomultiplier. Confocal microscope (Olympus, Tokyo, Japan) scanned images at 12 frames/min (~1 image every 5 s).

**Confocal Microscopy of Internalized CD38**—WT and C119K/C201E mutant-transfected HeLa cells were incubated with anti-CD38 antibody (1 μg/ml) for 30 min on ice, and the cells were washed with ice-cold HBSS. CD38 ligation was performed by incubation of cells with tetramethylrhodamine isothiocyanate-labeled Fab’₂, anti-mouse IgG for 0 or 30 min, and then the cells were washed and fixed with 3.7% paraformaldehyde. Both cells were centrifuged onto slides and visualized with a confocal microscope. The intracellular CD3PR levels were measured (d).

**Glucose Tolerance Test**—Female C57BL/KsJ-db/db mice 7 weeks of age were purchased from Clea Japan (Tokyo, Japan). Mice were maintained in an environmentally controlled room with a 12-h light-dark cycle and were allowed free access to diet and specific pathogen-free water. For glucose tolerance test, mice were fasted overnight and received single oral administration of various concentrations of OTC (8–80 mg/kg). Five h later, mice were injected with glucose (1 g/kg, intraperitoneally), and blood samples were taken at various time points (0–120 min). Blood glucose levels were measured by the glucose oxidase method using a glucose analyzer (Lifescan, Inc., Milpitas, CA), and
serum insulin levels were determined using a radioimmunoassay kit (Amersham Biosciences, Inc.). For the long term effect of OTC, mice received OTC orally twice a day with various concentrations of OTC (0–80 mg/kg/day). At 1, 2, and 4 weeks after OTC administration, mice were fasted overnight and were injected intraperitoneally with glucose (1 g/kg). Blood samples were taken at various time points (0–120 min), and blood glucose levels were measured.

Immunohistochemistry—Mice were anesthetized with ether, and an abdominal region was incised. Pancreases were then removed from the mice and fixed overnight in a cold 4% paraformaldehyde solution in PBS. Fixed tissues were processed routinely for paraffin embedding, and 5–6-μm sections were used for hematoxylin–eosin or immunohistochemical staining. The tissue sections for detection of insulin were rinsed three times with Tris-buffered saline before conjugation with antibodies. The sections were incubated for 3 h with guinea pig anti-insulin polyclonal antibody or rabbit anti-glucagon polyclonal antibody (DAKO Corp., Carpinteria, CA) diluted 1:50 in Tris-buffered saline containing 1.5% BSA. They were then incubated for 30 min with anti-guinea pig IgG-horseradish peroxidase conjugate (Advanced Biochemicals Inc., Chonju, Korea) as the secondary antibody, and reactivity was detected with 3-amino-9-ethylcarbazole. Nuclei were counterstained with hematoxylin. Positive staining morphology was analyzed using a microscope (Leica, Wetzlar, Germany).

RESULTS

Cys-119 and Cys-201 Play an Important Role in CD38 Internalization—We expected that C119K/C201E mutant should have normal or increased intracellular cADPR concentration ([cADPR]), because the ADP-ribosyl cyclase activity of C119K/C201E mutant was equal to the level of wild type CD38, whereas the cADPR hydrolase activity was lost (Fig. 1). However, the cysteine mutant-transfected cells showed completely reduced [cADPR], as compared with wild type CD38 (Fig. 1). This result led us to speculate that these cysteine residues might play an important role in the internalization of CD38 because CD38 internalization has been proposed as a mechanism by which the ectoenzyme produced intracellular cADPR (20). Ligation of CD38 with anti-CD38 antibodies has been shown to elicit cellular responses such as the internalization of CD38 and elevation in cytoplasmic Ca2+ (17). Thus, to examine the internalization of CD38 and signaling events that were occurring, we used the CD38 ligation in the transfected cells. When analyzed with FACSScan after CD38 ligation with anti-CD38 antibody, the C119K/C201E mutant did not show any internalization of CD38, whereas the wild type completely internalized (Fig. 2a). Next, we measured the [Ca2+]i following CD38 ligation in the Cys-119/201 mutant-transfected HeLa cells. CD38 ligation-induced increase of Ca2+ concentration was completely abolished in the mutant, whereas wild type CD38-transfected cells showed an increase of CD38 ligation-induced Ca2+ (Fig. 2b). Immunofluorescent confocal microscopic examination revealed CD38 on the cell surface as a ring shape in wild type-transfected cells, and the fluorescence on the cell surface disappeared on ligation of CD38 and appeared as intracellular clusters (Fig. 2c). On the other hand, C119K/C201E mutant showed no change of CD38 localization after ligation with anti-CD38 (Fig. 2c). These results suggested that Cys-119 and Cys-201 are essential for the internalization of CD38 to increase Ca2+ concentration by increasing intracellular cADPR. Thus, we measured intracellular cADPR level in the transfected cells before and after the treatment with anti-CD38 antibody and found CD38 ligation-induced increase of [cADPR], and basal [cADPR], in wild type-transfected cells, whereas we found complete reduction in the mutant cells (Fig. 2d). Taken together, our findings suggested that the disulfide bond between Cys-119 and Cys-201 in the CD38 molecule might be involved in the internalization of CD38.

Reduction of the Disulfide Bond between Cys-119 and Cys-201 Induces CD38 Dimerization—Reduction of the intramolecular disulfide bond formed between the Cys-119 and Cys-201 of CD38 may enhance the possibility of intramolecular disulfide bonding, resulting in dimer formation. Thus, we tested whether C119K or C201E mutation might influence dimer formation because mutants of a single mutation of C119K or C201E mutants still have a single reduced cysteine at 201 or 119. Indeed, C119K or C201E mutants showed dimer formation; Cys-119 and Cys-201 Induces CD38 Dimerization.
ent manner in the wild type transfected cells (Fig. 3b). In contrast, the C119K/C201E mutant did not form any dimer by treatment with the reducing agent (Fig. 3c). These results suggested that CD38 was dimerized through intermolecular disulfide bond at Cys-119 and Cys-201, which was prerequisite for its internalization. Furthermore, the new intermolecular disulfide bond should specifically be formed between Cys-119 and Cys-119 or between Cys-201 and Cys-201. To test the physiological relevancy of this hypothesis, we examined whether CD38 of isolated pancreatic islets could be dimerized by treatment with reducing agent, and we found that dithiothreitol at 1 mM concentration indeed induced dimerization of CD38 (Fig. 3d). Moreover, a physiological insulin secretagogue glucose at 25 mM could induce dimerization of CD38 (Fig. 3d). **Effect of L-2-Oxothiazolidine-4-carboxylic Acid on Insulin Release from Pancreatic Islet Cells**—Thiol compounds such as glutathione have been shown to induce the oligomerization and internalization of CD38 (21), suggesting that disulfide bonds of CD38 play a role in CD38/cADPR/Ca\(^{2+}\) signaling. To test the effect of reducing agent on CD38 internalization, we searched

![Fig. 4. Effect of OTC on insulin secretion, CD38 internalization and Ca\(^{2+}\) influx. a, dose dependence of OTC on insulin secretion in isolated islet cells. b, dose dependence of glucose on insulin secretion in isolated islet cells by OTC treatment. c, CD38 internalization induced by OTC treatment. CD38-GFP-transfected HEK293 cells were incubated with or without OTC and visualized with a confocal microscope. d, OTC-induced enhancement of [Ca\(^{2+}\)] in isolated islet cells. Arrows indicate the addition of glucose. e, CD38 expression in CD38 sense or antisense oligonucleotide-treated pancreatic islet cells. f, insulin secretion by the treatment of 1 mM OTC in CD38 sense or antisense oligonucleotide-treated pancreatic islet cells. Data represent means ± S.E. of triplicate sample.](image-url)
and found an ideal drug for the purpose. OTC, a prodrug of cysteine, is readily absorbed into the body and cleaved into cysteine by a ubiquitous intracellular enzyme, 5-oxoprolinase (22). Thus, intake of a sufficient amount of this drug may increase reducing power in the body, including pancreas (23).

To test the physiological relevance of the above notion, pancreatic islet cells were employed because cADPR is generated in pancreatic islet cells by glucose stimulation, thereby serving as a second messenger for Ca$^{2+}$/H$^{+}$ mobilization to secrete insulin (8).

Indeed, OTC induced insulin release from isolated mouse islets in a dose-dependent manner in the presence of 5.6 mmol/liter glucose (Fig. 4a). The stimulating effect of OTC (1 mM) was dependent on glucose concentration (Fig. 4b). Interestingly, however, OTC was ineffective on insulin release in the presence of 2.8 mmol/liter glucose (Fig. 4b). To examine whether CD38 internalization would be increased by the treatment of OTC, we used green fluorescent protein-fusion CD38-transfected HeLa cells. CD38 internalization was clearly increased in the OTC-treated cells in the presence of 5 mM glucose as compared with control cells (Fig. 4c). Furthermore, OTC-treated islet cells showed higher cytoplasmic Ca$^{2+}$ levels than the control islet (Fig. 4d). To further substantiate the conclusion that the effect of OTC was mediated via CD38, we constructed CD38 antisense oligonucleotide and tested whether the effects of OTC could be eliminated in CD38 antisense-treated cells. CD38 expression was found to be dramatically suppressed in the CD38 antisense-treated cells as compared with the CD38 sense-treated cells (Fig. 4e), and the antisense-treated cells showed complete reduction of insulin secretion by the treatment of OTC as well as a reduction in the basal level of insulin (Fig. 4f).

**Effect of OTC Intake on Insulin Release and Glucose Tolerance Test in db/db Mice**—Intraperitoneal glucose tolerance tests performed at 7 weeks of age in C57BL/KsJ-db/db mice after treatment with a single dose of OTC revealed that blood glucose levels were reduced in a dose-dependent manner (Fig. 5a) and that insulin secretion was increased (Fig. 5b). To investigate the long term effect of OTC, glucose tolerance tests were performed at 8, 9, and 11 weeks of age following 1, 2, and 4 weeks of OTC treatment. Glucose tolerance in those mice after OTC administration was ameliorated (Fig. 5c). Morphological examination of the mice treated with OTC for 4 weeks showed that the islet mass was larger, and insulin contents of the islets were higher in the treated group than those from the control group (Fig. 5d). Taken together, these data indicated that OTC improved glucose tolerance not only by enhancing insulin secretion but also by protecting pancreatic islets.
In this study, we showed that a prodrug of cysteine, OTC, could be used as an antidiabetic agent that induces insulin secretion by a novel mechanism; OTC induces CD38 internalization, thereby producing intracellular cADPR for subsequent Ca^{2+} signaling. Though physiological ligand(s) for CD38 has yet to be determined, our findings suggest that the cellular reducing system is a physiologic ligand for CD38 to produce intracellular cADPR and to generate intracellular Ca^{2+} signaling. Ligand-induced internalization of CD38 has been observed in a variety of cells (20) and has been proven to be associated with cADPR-dependent [Ca^{2+}]i increases (17). A proposed mechanism by which a topological problem of the ectocellular active site of CD38/intracellular localization of cADPR receptor could be overcome was suggested by the demonstration of a specific transport system for NAD in plasma membrane and of CD38 itself as a cADPR transporter (20, 24). Whatever the mechanism, the prime prerequisite for intracellular production of cADPR by CD38 is its internalization, as shown in this study.

Aplysia cyclase was cloned and recombinantly expressed, and its three-dimensional structure was determined (16). The three-dimensional picture predicts that the amino acid residues corresponding to cysteine 119 and cysteine 201 of human CD38 are located on the outer surface of the enzyme, linked by a disulfide bond. This structural finding is consistent with our data showing that cysteine 119 and cysteine 201 of CD38 are essential sites for its internalization and that a reducing agent enhances CD38 internalization. Data on the cyclase mutation with blockages of CD38 internalization and intracellular cADPR production (Fig. 2, c and d) indicated that some reducing power to break the disulfide bond between cysteine 119 and cysteine 201 seemed to be essential for CD38 internalization. This assumption was supported by a finding that GFP-CD38-expressed cells cultured in serum-containing media showed a diffuse fluorescence in cytosol, whereas the cells incubated with serum-free media had fluorescence only on the plasma membrane (data not shown). Although the exact mechanism by which reducing power induces CD38 internalization is yet to be determined, one of the explanations would be that reduction of the cysteine 119-cysteine 201 disulfide bond in a CD38 molecule would favor formation of the intermolecular disulfide bond between different CD38 molecules, producing dimerization (or oligomerization) of CD38 (21). Receptor dimerization (or oligomerization) has been well established to be prerequisite for subsequent internalization (25). Alternatively, breakage of the disulfide bond between cysteine 119 and cysteine 201 itself may result in a conformational change of CD38 to induce its internalization, as predicted previously from the data of the crystal structure of Aplysia cyclase (16).

Cysteine is a precursor for the synthesis of glutathione, a major cellular reducing power (26). It is generally believed that cysteine is a rate-limiting precursor for GSH synthesis and that intracellular GSH level is regulated by the availability of cysteine. Administration of cysteine is severely restricted because of its rapid oxidation to cystine, which has an extremely low solubility. Cysteine in moderate amounts is also toxic due to the deleterious effect of hydrogen peroxide formed during its oxidation to cystine (27). On the other hand, OTC, a substituted thiazolidine derivative, is apparently nontoxic and is readily transportable into cells where 5-oxoprolinase, a ubiquitous intracellular enzyme, catalyzes ATP-dependent conversion of OTC to l-cysteine (22). OTC is also a prodrug of cysteine in humans and raises the plasma concentration of cysteine and the intracellular concentrations of cysteine and GSH in lymphocytes of healthy volunteers (28–30). Because GSH functions in the reduction of the disulfide linkages of proteins and intracellularly synthesized GSH is transported out of cells to be a main source of plasma glutathione (31), reduction of the disulfide bond between cysteine 119 and cysteine 201 of CD38 is expected to be enhanced by increased GSH concentration on the cell membrane as well as in the surrounding environment following the administration of OTC.

Other investigators (32–38) showed that high glucose-induced deterioration of pancreatic β-cells due to oxidative stress and antioxidants such as N-acetylcysteine could exert beneficial effects on pancreatic β-cells by neutralizing the toxic effect of oxidative stress. In support of the above, OTC might have influenced islet cells through its direct action, as presently shown by the preservation of cell mass and insulin content in islets (Fig. 4d). Nevertheless, our data indicate that the major antidiabetic action of OTC may be attributable to its insulinotropic effect, which reduces blood glucose levels, although we cannot exclude a possibility that the antioxidant may exert its effect directly on target tissues as well as pancreatic islet cells. Therefore, from the above considerations, we suggest that OTC can be of therapeutic use in diabetes by improving glucose tolerance and by reducing glucose toxicity.

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