

Direct Evidence for Glucose Consumption Acceleration by Carbonates in Cultured Cells

Kenji Sorimachi

Educational Support Center, Dokkyo Medical University, Miub, Tochigi 320-0293, Japan, and Research Laboratory, Gunma Agriculture and Forest Development, Takasaki, Gunma 370-0851, Japan.

ABSTRACT

Established Py-3Y1-S2 rat fibroblast cells were used to evaluate whether NaHCO₃ or Na₂CO₃ influences glucose metabolism in vitro, because factors that contribute to metabolic pathways are much simpler to be evaluated in cultured cells than in whole animal bodies. The effects of the carbonates on glucose consumption decreased at high concentrations, >5 mg/ml for Na₂CO₃ and >7 mg/ml for NaHCO₃, because of the increased pH of the culture medium. The effects of the carbonates on glucose consumption were additive with those of vanadium and concanavalin A. Streptozotocin, alloxan, and nicotinamide, which induced diabetes in animals, reduced glucose consumption by Py-3Y1-S2 cells, and the inhibitory effects of these reagents were abolished by both Na₂CO₃ and NaHCO₃. Finally, the carbonates increased lactate production from glucose in the cells, followed by the acceleration of lactate secretion into the culture medium. The present study clarified that NaHCO₃ and Na₂CO₃ directly regulated glucose metabolism. *Key Words: Glucose, Metabolisms, Diabetes, Anti-diabetic, Carbonates (NaHCO3, Na2CO3), Concanavalin A, Vanadium.*

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INTRODUCTION

The prevalence of diabetes mellitus (DM) is increasing worldwide. The International Diabetes Federation reported that there were 425 million adults with diabetes in the world in 2017 [1]. The symptoms of chronic high blood sugar in DM included frequent urination, increased thirst, and increased hunger. If left untreated, DM can lead to serious complications like cardiovascular disease, stroke, chronic kidney failure, foot ulcers, and eye damage. Insulin via injections is used for treatment of type 1 DM, while insulin and several oral drugs that inhibit glucose production from polysaccharides are used for type 2 DM treatment. However, these drugs only treat the symptoms, and there is no basic remedy for DM. Previous studies have investigated whether insulin plays a role in memory formation in vivo [2], and whether insulin contributes to dementia, particularly Alzheimer's disease [3-5].

Regarding its mode of action as a peptide hormone, insulin binds to its receptor on the plasma membrane,

leading to intramolecular phosphorylation within the activated receptor as a tyrosine kinase. The signal transduction proceeds through phosphatidylinositol-3kinase, protein kinase B, and glucose transporter-4 (GLUT-4), with GLUT-4 plus K⁺ accelerating glucose uptake into cells [6]. Vanadium compounds have been reported to exhibit insulin-like activity not only in vitro [7, 8], but also in vivo [9-16], and several vanadium compounds have been investigated for their insulin-like activity [17-21]. The insulin-like effects of vanadates are based on inhibition of protein-tyrosine phosphatase [22]. However, to the knowledge of researchers, suitable vanadium compounds have not yet been developed as anti-DM drugs because of the serious cytotoxic effects of high vanadium concentrations. It has been suggested that Mt. Fuji subsoil water filtered through basalt can exhibit insulin-like activity, because water contains vanadium pentoxide (V₂O₅) in vivo [23]. Recently, it has been confirmed that Mt. Fuji subsoil water accelerates glucose consumption in vitro using established Py-3Y1-S2 rat fibroblast cells [24] and human primary fibroblasts [25].

E-mail: keniis@icom.home.ne.ip

Corresponding author: Kenji Sorimachi

Address: Research Laboratory, Gunma Agriculture and Forest Development, Takasaki, Gunma 370-0851, Japan.

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Vanadium pentoxide is soluble in the alkaline condition, but its water solubility is quite low (0.7-0.8 g/L). Indeed, the pH value of commercial Mt. Fuji subsoil water (Healthy Vana Water) containing 130 µg/L vanadium was 8.3 [24]. If vanadium-containing water can be prepared by mixing a small amount of Mt. Fuji basalt powder with normal water, the vanadium-containing water could be conveniently used instead of Mt. Fuji subsoil water.

Associations between metabolic acidosis, insulin resistance, and cardiovascular risks have been reported since 1924 [26]. Although the correction of metabolic acidosis by nutritional therapy and/or oral administration of sodium bicarbonate was widely applied in chronic kidney disease [27-29], it remained unknown whether metabolic acidosis reduced insulin resistance and/or improved insulin effects on target cells in diabetic subjects. Bellasi et al. [30] evaluated whether metabolic acidosis correction by sodium bicarbonate administration can improve peripheral endogen insulin utilization by target organs in diabetic subjects with chronic kidney disease treated with oral antidiabetic drugs. They reported that sodium bicarbonate administration improved insulin resistance in chronic kidney disease at the serum glucose and insulin levels. However, they commented that further investigations were required to evaluate their results in diabetic and non-diabetic chronic kidney disease patients. Indeed, another group reported that sodium bicarbonate treatment did not improve insulin sensitivity and glucose control in non-diabetic older adults [31]. These contradictory findings seemed to arise from different experimental systems investigated with many different factors. The present study was designed to clarify whether carbonates (NaHCO3 and Na2CO3) can directly influence glucose metabolism in simple cultured cells.

MATERIALS AND METHODS

Cell culture

Py-3Y1-S2 rat fibroblast cells were cultured in Dulbecco's minimum essential medium (DMEM) containing 5% fetal bovine serum (Gibco, Thermo Fisher Scientific K.K., Tokyo, Japan) in the presence of 5% CO₂ at 37°C. Stock cells cultured in 25-cm² plastic culture flasks were trypsinized and inoculated into 24-well culture plates. After the cells had almost reached confluent monolayers, they were cultured in the presence or absence of NaHCO3, Na2CO3, and other chemical compounds like vanadium, concanavalin A (Con A), nicotinamide, streptozotocin (STZ), and alloxan monohydrate.

Glucose assay

Glucose concentrations in culture media were measured by a previously reported method [24] using a Glucose CII-test (Wako Pure Chemical, Osaka, Japan). For this, a $10-50-\mu$ l aliquot of culture medium was mixed with the assay solution (0.5 ml), and the absorbance at 505 nm was measured after 15 min. To express the effects of reagents on glucose consumption, the amount of consumed glucose in the sample medium was divided by that in the control medium (Milli-Q water) without a test reagent.

Lactate assay

Lactate concentrations in culture media were measured with a Lactate Assay Kit-WST (Dojin Chemicals, Tokyo, Japan) according to the manufacturer's protocol. Briefly, a 20- μ l aliquot of sample solution was mixed with 80 μ l of working solution, and incubated at 37°C for 30 min. After the enzyme reaction, the absorbance at 450 nm was measured to calibrate the lactate concentration.

Protein assay of cultured cells

Cellular protein assays were carried out using a previously reported method [24]. Cells cultured in 24well plates were washed with phosphate-buffered saline and treated with 0.2 ml of Mammalian Protein Extraction Reagent (Thermo Scientific, Rockford, IL, USA). An aliquot of the solubilized cell solution (4 μ l) was added to 156 μ l of H₂O, and mixed with Bio-Rad Protein Assay Dye Reagent (Bio-Rad Laboratories, Hercules, CA, USA). The absorbance at 595 nm was measured. Bovine serum albumin was used as a standard.

Chemicals

DMEM powder (Misui, Tokyo, Japan) was dissolved in Milli-Q water, and autoclaved. The medium (1 L) was neutralized by adding 4 ml of 10% NaHCO₃, according to the technical protocol recommended by the manufacturer. NaHCO₃, Na₂CO₃, vanadium, nicotinamide, STZ, and alloxan monohydrate were purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan). Con A was obtained from Sigma Chemical Co. (St. Louis, MO, USA).

Statistical analysis

Statistical calculations by the *t*-test were performed using Microsoft Excel (version 2010). Values of p < 0.05 and p < 0.01 were considered significant and highly significant; respectively.

RESULTS AND DISCUSSION

Effects of NaHCO3 and Na2CO3

To prepare vanadium-containing water, Mt. Fuji basalt powder (1–2- μ m diameter) was treated with 1 mg/ml NaHCO₃, which is safe for the human body, and this water was used to make the cell culture medium. The cell culture medium containing Mt. Fuji basalt-treated water increased glucose consumption in cultured cells (Fig. 1). However, an increase in Mt. Fuji basalt powder for water treatment from 5 mg/10 ml to 200 mg/10 ml did not cause any further increase in glucose consumption by cultured cells. These findings indicated that Mt. Fuji basalt powder treatment may not be involved in acceleration of glucose consumption by cultured cells. Indeed, only NaHCO₃ or Na₂CO₃ accelerated glucose consumption by cultured cells (Fig. 1).



Figure 1. Effects of basalt powder on glucose consumption by cultured Py-3Y1-S2 cells in the presence of NaHCO₃ or Na₂CO₃. Mt. Fuji basalt powder with ~2-μm diameter was kindly supplied by Mt. Fuji Yogan Institute (Yamanashi, Japan), and 5 or 200 mg of powder was treated with 1 mg/ml NaHCO₃ for 30 min at room temperature. After treatment with NaHCO₃, the solution was centrifuged at 3,000 rpm for 10 min, and the supernatants were used for preparation of culture media. Data represented means ± SD of 3 or 13 independent experiments. *p < 0.05; **p < 0.01.

Regarding the NaHCO₃ concentration, the effect of NaHCO3 was significant at 1 mg/ml, and subsequently increased in a dose-dependent manner up to 5 mg/ml. Thereafter, the effect of NaHCO₃ gradually decreased in the range of 7-10 mg/ml (Fig. 2A). For Na₂CO₃, a significant effect was observed at 0.5 mg/ml, and the maximum effect (~200% of control) was observed at 2 mg/ml (Fig. 2B). The latter concentration was close to the serum bicarbonate concentrations (2.0-2.4 mg/ml) that improved serum glucose and insulin levels in diabetic chronic kidney disease patients with acidosis after oral administration of bicarbonate in vivo [30]. The effect of Na₂CO₃ decreased rapidly in the range of 4–5 mg/ml, and completely inhibited glucose consumption by cultured cells at >7 mg/ml. Both carbonates accelerated glucose consumption in TE-13 cells derived from human esophageal cancers [32] (data not shown).





Cellular proteins. NaHCO₃ and Na₂CO₃ solutions are alkaline, and their pH values in culture media were 8.0-9.5 in the presence of 5% CO₂ in the cell incubator. Of course, culture medium initially contained NaHCO₃ to maintain the pH at around 7.5. The cellular protein





Figure 3. Effect of NaHCO₃ (A) or Na₂CO₃ (B) on cellular protein. Data represent means ± SD of 5 independent experiments. *p < 0.05; **p < 0.01.

contents of Py-3Y1-S2 cells were independent of NaHCO₃ in the range of 0.5-8 mg/ml, while NaHCO₃ at 10 mg/ml reduced the cellular protein to 70% of the control (Fig. 3). The decrease in cellular protein content was linked with the disappearance of glucose consumption acceleration at the high concentration of NaHCO₃ (Fig. 2A). Similarly, the Py-3Y1-S2 cellular protein content was markedly reduced by 5 mg/ml Na₂CO₃ to around 30% of the control (Fig. 2B). The cellular protein content was reduced to <10% in the presence of >7 mg/ml Na₂CO₃. This cytotoxic effect of Na₂CO₃ was in accordance with the decrease in glucose consumption (Fig. 3), and was caused by the high pH of 9.5 in the absence of CO₂. The culture medium containing 5 mg/ml Na₂CO₃ had a pH of ~8.5 after incubation in the presence of 5% CO₂. Therefore, conditions involving high pH above 9 led to cell death.

Additive effects of Con A or vanadium

The lectin Con A was reported to exert insulin-like effects during *in vitro* experiments [33]. It was shown that Con A shared insulin receptors with insulin by *in vitro* experiments involving binding assays of labeled Con A and insulin [34, 35].

When cultured Py-3Y1-S2 cells were treated with Con A at 0.025 mg/ml, glucose consumption increased in a dosedependent manner (Fig. 4). Con A at 0.1 mg/ml further increased glucose consumption, and the effect almost reached a plateau. Thus, the effects of Con A at 0.1 mg/ml were examined at different NaHCO₃ concentrations. NaHCO3 at 0.5 mg/ml and 1.0 mg/ml further increased glucose consumption with Con A at 0.1 mg/ml, while the effect of NaHCO3 at 5.0 mg/ml was almost null in the presence of 0.1 mg/ml Con A (Fig. 5A). Similarly, Na₂CO₃ at 0.5 mg/ml further increased glucose consumption with Con A at 0.1 mg/ml (Fig. 5B). These results indicated that combinations of NaHCO₃ or Na_2CO_3 with Con A showed sufficient effects even low concentrations compared with their use alone.



Figure 4. Effect of Con A on glucose consumption by Py-3Y1-S2 cells. Data represent means ± SD of 5 independent experiments. *p < 0.05; **p < 0.01.

In a previous study [24], vanadium accelerated glucose consumption in a dose-dependent manner in the range of $25-10,000 \mu g/L$, while an inhibitory effect was observed at >1.0 mg/L in Py-3Y1-S2 cells. When cells were cultured with 1.0 mg/L vanadium plus 1.0, 2.5, or 5.0 mg/ml NaHCO₃, glucose consumption was significantly accelerated in the presence of 1.0 and 2.5 mg/ml NaHCO₃ (Fig. 5C). However, no additive effect of vanadium was observed at 5.0 mg/ml NaHCO₃. Similarly, lower concentrations of Na₂CO₃, 0.5 mg/ml and 1.0 mg/ml, exhibited a significant additive effect with vanadium, while 5.0 mg/ml Na₂CO₃ had no additive effect with vanadium (Fig. 5D).

Effects of diabetic reagents

STZ, alloxan, and nicotinamide are known chemical compounds that can induce diabetes in animals [36, 37]. In animals, alloxan destroys β -cells in the pancreas at high concentrations, and induces type 2 diabetes at low concentrations. When Py-3Y1-S2 cells were cultured in the presence of the above drugs *in vitro*, the glucose consumption in the culture medium was significantly reduced (Fig. 6A). In the present study, the drugs did not cause significant reductions in cellular protein amounts (data not shown). In contrast, addition of NaHCO₃ or Na₂CO₃ to the culture medium abolished the effects of STZ.











Figure 5. Effect of vanadium or Con A on glucose consumption by Py-3Y1-S2 cells in the presence of NaHCO₃ (A and B) or Na₂CO₃ (C and D). Data represent means ± SD of 5 independent experiments. *p < 0.05; **p < 0.01.

alloxan, and nicotinamide in Py-3Y1-S2 cells (Fig. 6B). These findings indicated that the reduction in glucose consumption by Py-3Y1-S2 cells was not caused by cell death.

Lactate production

When PY-3Y1-S2 cells were cultured in the presence of NaHCO₃ or Na₂CO₃, the concentration of lactate in the culture medium was significantly increased (Fig.7). Further addition of NaHCO₃ or Na₂CO₃ to the culture medium accelerated lactate secretion by the cells. These findings indicated that carbonates directly regulated glucose metabolism in Py-3Y1-S2 cells. Meanwhile, STZ, alloxan, and nicotinamide significantly reduced lactate production, and further addition of NaHCO₃ or Na₂CO₃ abolished the effects of these drugs.





Figure 6. Effect of NaHCO₃ or Na₂CO₃ on glucose consumption by Py-3Y1-S2 cells and on cellular protein amounts in the presence of STZ, alloxan, or nicotinamide. The concentration of each reagent was 1 mg/ml. The vertical axes represent glucose consumption (A), and cellular protein amounts (B). Data represent means ± SD of 3 independent experiments. *p < 0.05; **p < 0.01.



Figure 7. Effect of NaHCO₃ or Na₂CO₃ on lactate production by Py-3Y1-S2 cells in the presence of STZ, alloxan, or nicotinamide. The vertical axis represents lactate concentration. Data represent means \pm SD of 6–8 independent experiments. *p < 0.05; **; p < 0.01.

Glucose is metabolized to pyruvic acid during the first stage in the absence of oxygen, and then alcohols or organic acids are formed via the TCA cycle based on various enzyme reactions in cells. During the second stage in the presence of oxygen, pyruvic acid is finally converted to CO_2 and H_2O . Thus, NaHCO₃ or Na₂CO₃ formed from CO₂ could be involved in glucose regulation like insulin. These regulatory mechanisms by carbonates resemble the autocrine or paracrine mechanisms of cytokines.

The basic structure of both NaHCO₃ and Na₂CO₃, which accelerated glucose consumption, was a carbonyl group, =C=O, while the structures of STZ, alloxan, and nicotinamide, which induced type 1 diabetes and reduced glucose consumption in cultured cells, also contained a carbonyl group. The carbonyl group of diabetes-inducing reagent like STZ, alloxan, and nicotinamide was a part of an amide structure, -CONHR. Ceramides consisting of an amino group-bound carbonyl group were shown to induce insulin resistance [38]. However, amino acids containing a carboxyl group did not affect glucose uptake in the present study, although their structures contained a carbonyl group that was not bound to an amino group. These findings indicated that glucose consumption by NaHCO₃ or Na₂CO₃ was controlled by side groups that bound to the carbonyl group, such that the $-O^-$ accelerated glucose consumption and the -N- reduces glucose consumption.

 $NaHCO_3$ is used as baking soda in daily life and as a medicine for alkaloids. However, because $NaHCO_3$ and Na_2CO_3 are alkaline chemical compounds, their concentrations for usage should be carefully controlled. The present results may contribute to the development of alternative medicines as well as new medicines for DM and dementia.

CONCLUSIONS

Two carbonates, NaHCO₃ and Na₂CO₃, directly accelerated glucose consumption in established Py-3Y1-S2 rat fibroblast cells. Combinations of the carbonates with vanadium or Con A further increased glucose consumption, while combinations of the carbonates with STZ, alloxan, or nicotinamide abolished the effects of the drugs in Py-3Y1-S2 cells. Both carbonates increased lactate production by Py-3Y1-S2 cells. Taken together, the present study indicated that NaHCO₃ and Na₂CO₃ directly regulated glucose metabolism in Py-3Y1-S2 cells.

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Conflict of Interest

The present work has been used for the application of patents.

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