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Fermented milk, Kefram-Kefir enhances glucose uptake into insulin-responsive muscle cells

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Abstract

Diminution of insulin-responses in the target organ is the primary cause of non-insulin dependent diabetes mellitus (NIDDM). It is thought to be correlated to the excessive production of reactive oxygen species (ROS). In this article, we attempted to evaluate whether fermented milk, Kefram-Kefir known as an antioxidant, reduces the cellular ROS levels and can stimulate the glucose uptake in L6 skeletal muscle cells. Water-soluble or chloroform/methanol-extracted fractions from Kefram-Kefir were examined to evaluate the glucose uptake ability of L6 myotubes. As a result, the water-soluble fraction augmented the uptake of glucose in L6 myotubes both in the presence and absence of insulin stimulation. Estimation of intracellular ROS level revealed that the water-soluble fraction of Kefram-Kefir reduced the intracellular ROS level on both the undifferentiated and differentiated L6 cells. Especially, glucose uptake was augmented up to six times with the addition of water-soluble fraction in the insulin-stimulated L6 myotubes. Glucose transport determination revealed that the active agent in Kefram-Kefir was resistant to autoclave and stable in pH range from 4 to 10, and the small molecule below the molecular weight of 1000. Furthermore, this augmentation was inhibited in the presence of phosphatidylinositol 3-kinase (PI 3-kinase) inhibitor wortmannin. Considering together with the reports that PI 3-kinase is located in the insulin signaling pathway and the participation in the translocation of glucose transporter 4 to the cell membrane, it is suggested that the water-soluble fraction of Kefram-Kefir activates PI 3-kinase or other upstream molecules in the insulin signaling pathway, which resulted in the augmentation of glucose uptake and its specific inhibition by wortmannin.

Abbreviations: DCF, 2′,7′-dichlorofluorescein; DCFH-DA, 2′,7′-dichlorofuluorescin diacetate; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; GLUT, glucose transporter; HEPES, *N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulfonic acid; IDDM, insulin-dependent diabetes mellitus; IR, insulin receptor; IRS-1, insulin receptor substrate-1; NIDDM, non-insulin-dependent diabetes mellitus; PI 3-kinase, phosphatidylinositol 3-kinase; ROS, reactive oxygen species.

Introduction

Diabetes mellitus is classified into two types, insulin dependent diabetes mellitus (IDDM) and non-insulin dependent diabetes mellitus (NIDDM). Both types of diabetes mellitus are undergoing failure in the proper control of circulating blood glucose concentration. Resulting hyperglycemia is associated with various fatal diseases such as cardiovascular disease (Keen et al., 1999; Manson et al., 1991; Reaven et

al., 1991), renal failure (Ritz et al., 1999), diabetic neuropathy (Vinik et al., 2000) and diabetic retinopathy (Ferris, 1993). In healthy humans, blood-sugar level is kept in concentration at around 5 mM. This mechanism is maintained by a well-known hormone, insulin, which stimulates blood glucose uptake into the muscles and adipocytes. The dysfunction of insulin secretion from the pancreas resulted in IDDM and the lowered response to insulin resulted in NIDDM. In the NIDDM patients, insulin-stimulated glucose uptake into muscle cells was reduced in both *in vivo* (DeFronzo et al., 1992) and *in vitro* (Dohm et al., 1988; Andreasson et al., 1991).

Recent participation of reactive oxygen species (ROS) has been noted in both IDDM and NIDDM (Rudich et al., 1997, 1998). Increased oxidative stress has been recognized to play a role in the occurrence and development of diabetes mellitus (Baynes et al., 1991; Wolff et al., 1991). Therefore, the utilization of antioxidant for therapeutic approaches such as α-tocopherol (Ceriello et al., 1992), ascorbic acid (Paolisso et al., 1994) or α-lipoic acid (Estrada et al., 1996) has focused.

Kefram-Kefir is fermented-milk which originated in the Caucasus Mountains, and is known to have numerous benefits such as anti-cancer effects (Kubo, 1992), stimulation of the immune system (Osada et al., 1994; Kabayama et al., 1997) and anti-oxidizing activity (Nagira, 1998; Kusumoto, 2002). Since oxidative damage in the insulin signaling pathway, such as hyperoxia (Bashan et al., 1992; Ceriello et al., 1996) or high glucose (Müller et al., 1991), etc., has been reported, we have tried to improve glucose uptake by shifting the redox state of muscle cells to a more reduced one by Kefram-Kefir.

Materials and methods

Cell culture

L6 cells (JCRB9081) derived from a rat skeletal muscle were obtained from the Health Science Research Resources Bank (Osaka, Japan), and cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with a 10% fetal bovine serum (FBS, Biowhittaker, Walkersville, MD) in a humidified atmosphere of 5% CO_2/95% air at 37 °C. Differentiation from myoblasts into insulin-responsive myotubes was performed by culturing them in 2% FBS/DMEM for ten days with fresh medium fed in two day intervals (Estrada et al., 1996).

Preparation of Kefram-Kefir sample

Kefram-Kefir was supplied by Nihon Kefir Co., Ltd. The centrifuged-supernatant from the Kefram-Kefir mixture was used as a Kefram-Kefir water-soluble fraction. The fraction extracted from the dried precipitates with 10 volumes of chloroform/methanol (2:1) was used as a Kefram-Kefir chloroform/methanol extracted fraction (Osada et al., 1994). The fraction was recovered by the evaporator and dissolved in ethanol for glucose transport determination. Further investigations of water-soluble fraction were performed by dialysis (M.W. cut off: 1000), gel filtration with Bio-Gel P-2 (Bio-Rad, Hercules, CA), and ion-exchange chromatography with TSK-gel DEAE-Toyopearl 650M (Tohso, Tokyo, Japan).

Glucose transport determination

Glucose Transport determination was carried out in L6 myotubes which differentiated into skeletal muscle, cells from myoblasts. The L6 myotubes were incubated in the culture media supplemented with 25% (v/v) Kefram-Kefir water-soluble fraction (final concentration: 9.6 mg ml^{-1}) or 10 μg ml^{-1} of Kefram-Kefir chloroform/methanol extracted fraction for 4 h at 37 °C, respectively. After incubation with or without 100 nM of insulin for 1 h, cells were rinsed twice with glucose-free *N*-2-hydroxyethylpiperazine-*N*''-2-ethanesulfonic acid (HEPES)-buffered saline (140 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 20 mM HEPES pH 7.4). The cells were incubated in HEPES buffered saline containing 1 μCi ml^{-1} of 2-deoxy-D-[^{3}H] glucose (Amersham Pharmacia Biotech, Buckinghamshire, U.K.) for 10 min. After the uptake period, the radioactive solution was aspirated and the cells were rinsed three times with ice-cold 150 mM NaCl, disrupted with the 0.5 N NaOH and neutralized with 2 N acetic acid and the incorporated radioactivity was determined by a liquid scintillation counter. Within each uptake experiment at least triplicate assays of each condition were performed.

Assay of intracellular redox state

Intracellular redox state levels were measured using a fluorescent dye, 2′,7′-dichlorofluorescin diacetate (DCFH-DA, purchased from Wako Pure Chemical Industries, Osaka, Japan) (Bass et al., 1983). L6 myoblasts or myotubes were treated 8 μM of DCFH-DA for 3 min after culture with each sample. Then fluorescent intensities of 2′,7′-dichlorofluorescein (DCF)

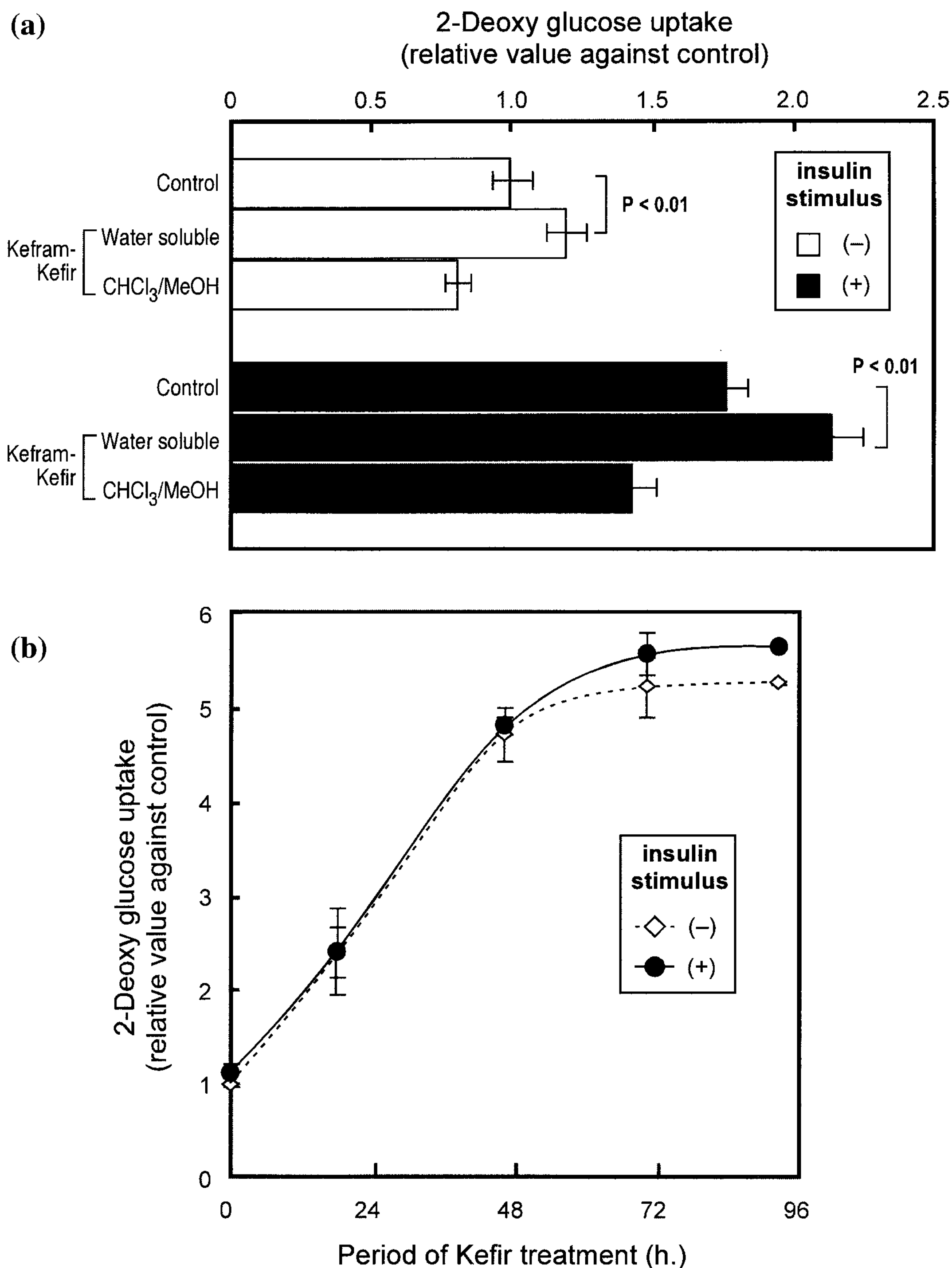

Figure 1. Effects of Kefram-Kefir on glucose uptake into L6 myotubes. (a) Effects of water-soluble and chloroform/methanol extracted Kefram-Kefir fractions on glucose uptake into L6 myotubes. L6 myotubes were cultured with the medium supplemented with 25% (v/v) of water-soluble Kefram-Kefir fraction (Control as equal volume of HEPES buffered saline) or 10 $\mu g\ ml^{-1}$ of chloroform/methanol extracted Kefram-Kefir fraction (Control as equal volume of ethanol) for 4 h, then the glucose transport determination was performed. (b) Time dependency of glucose uptake enhancement into L6 myotubes by water-soluble fraction. L6 myotubes were cultured with the medium supplemented with 25% (v/v) of water-soluble Kefram-Kefir fraction, and then the glucose transport determination was performed. Significant difference ($P < 0.01$) compared with the control.

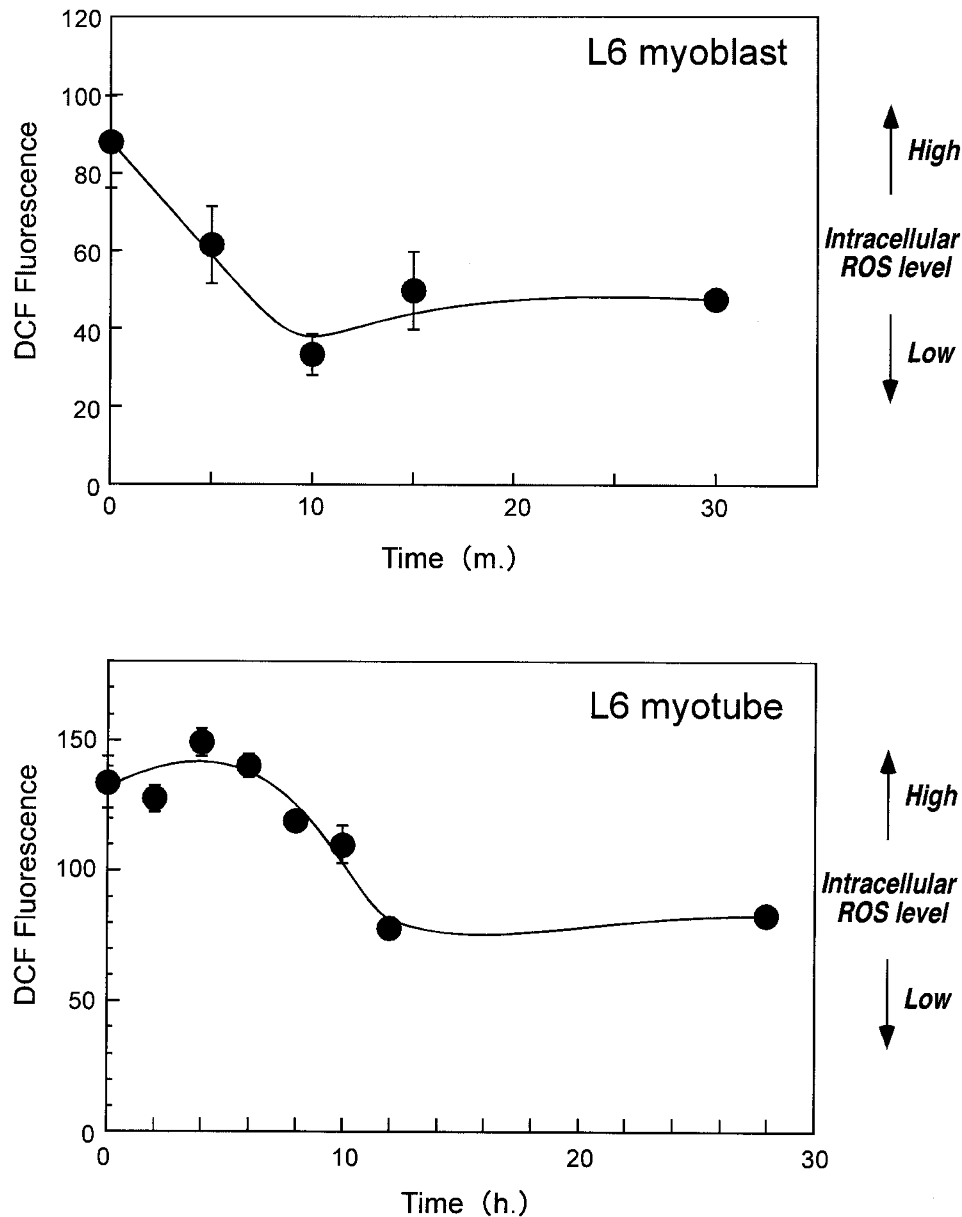

Figure 2. Effects of Kefram-Kefir on intracellular ROS level of L6 myoblasts and myotubes. Both cells were treated 8 μM of DCFH-DA for 3 min after culture with each sample. Then fluorescent intensities were measured by a confocal laser microscopy (Ex: 488 nm, Em: 525 nm).

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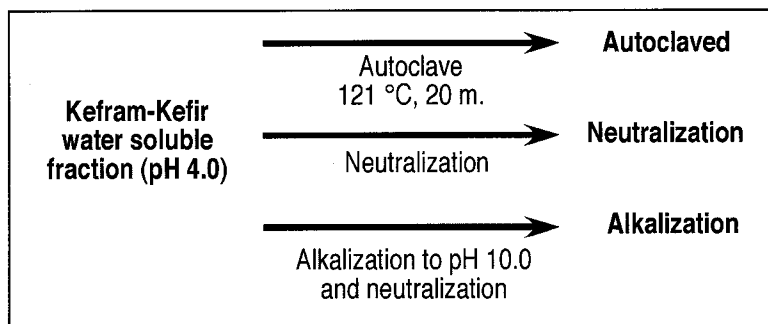
Downregulation of the insulin signaling pathway with wortmannin

The L6 skeletal muscle cells were treated with 0.1 μM or 0.5 μM of wortmannin (Wako) for 24 h at 37 °C, to inhibit PI 3-kinase which was located in the insulin signaling pathway (Okada et al., 1994). Then the glucose transport determination was performed.

Statistical analysis

Statistical analysis was performed using either paired *t*-test. Probabilities of 0.05 or less were considered to be statistically significant.



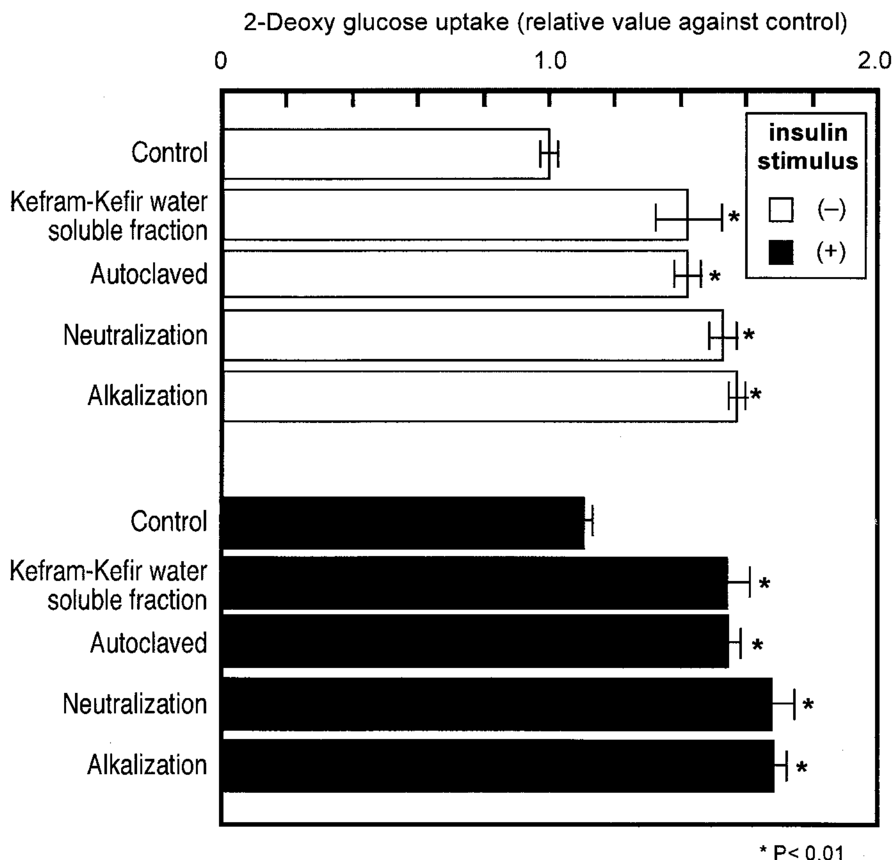


Figure 3. Stability of the water-soluble fraction in a high temperature and a pH range from 4 to 10. L6 myotubes were cultured with the media supplemented with 25% (v/v) of each samples for 4 h, then the glucose transport determination was performed. * Significant difference ($P < 0.01$) compared with the control.

Results

Kefram-Kefir water-soluble fraction enhanced glucose uptake in L6 myotubes

The insulin-responsive differentiated rat L6 myotubes were treated with the water-soluble or chloroform/methanol-extracted fractions of Kefram-Kefir for 4 h and then the glucose uptake abilities were evaluated. As shown in Figure 1a, the water-soluble fraction augmented the glucose uptake in L6 skeletal muscle cells. This enhancement was observed in both with insulin stimulation or not. However, chloroform/methanol-extracted fraction showed inhibitory effects. Therefore, we focused on the water-

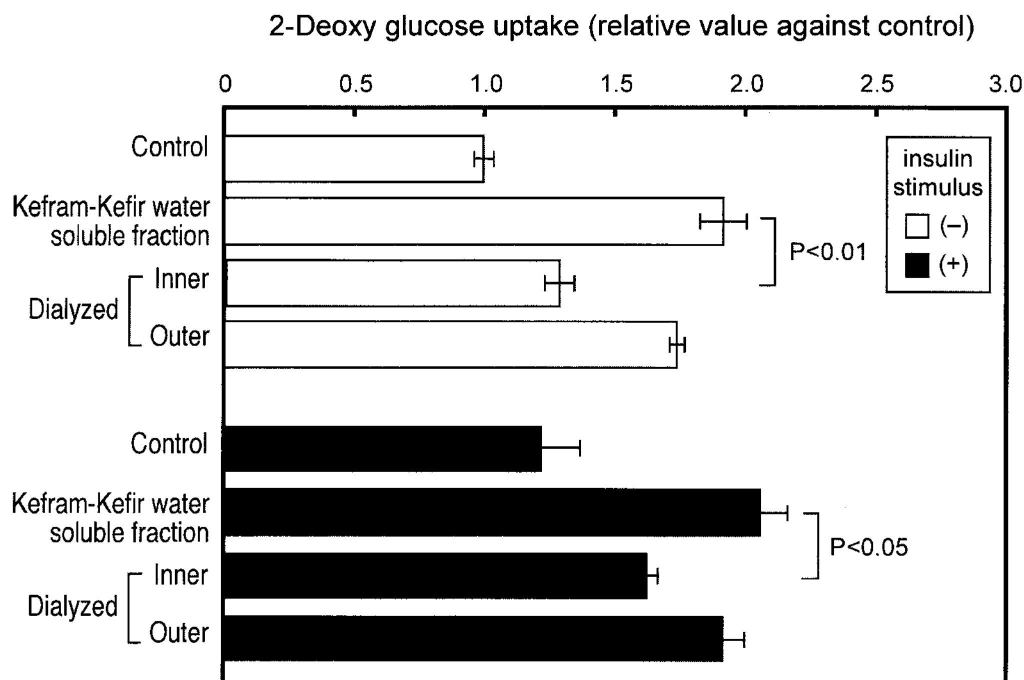


Figure 4. Effects of dialysis against glucose uptake augmentation of Kefram-Kefir. L6 myotubes were cultured with the media supplemented with 25% (v/v) of each samples for 4 h, then the glucose transport determination was performed. Significant difference ($P < 0.01$ or $P < 0.05$) compared with the treatment of Kefram-Kefir water-soluble fraction.

soluble fractions in the next investigation. The enhancing effect of Kefram-Kefir water soluble fraction was observed in the glucose transporter (GLUT) specific glucose uptake from the results of the GLUT-independent glucose uptake into cells assessed by treatment of cytochalasin B (data not shown). The water-soluble fraction enhanced the glucose uptake in L6 myotubes up to six times with a long period treatment (up to 72 h) (Figure 1b).

Kefram-Kefir reduced the intracellular ROS level

To evaluate the anti-oxidative effects of Kefram-Kefir on L6 cells, we estimated the intracellular ROS levels by a fluorescent dye, DCFH-DA. DCFH-DA incorporated cells reacted with intracellular hydrogen peroxide, producing DCF, a fluorescent substance with excitation at 488 nm and emission at 525 nm. This reagent was often used to investigate the intracellular ROS levels. The water-soluble fraction reduced the intracellular ROS levels on both the undifferentiated and the differentiated L6 cells (Figure 2). As shown in Figure 2, the ROS level of L6 myoblasts responded to earlier than myotubes. It was considered that exponential growing cells had a higher basal ROS level than differentiated ones. The results suggested that the anti-oxidative effect of Kefram-Kefir water-soluble fraction was one of the factors for glucose uptake enhancing in L6 myotubes.

Characterization of active agent in Kefram-Kefir to enhance the glucose uptake

Since the water-soluble fraction of Kefram-Kefir was around pH 4, neutral (pH 7) and alkalized (pH 10) samples were prepared by NaOH. The agent in the water-soluble fraction responsible for glucose uptake enhancing activity was stable in pH 4–10 (Figure 3). In addition, the activity of glucose uptake enhancing could not be lost by autoclaving (121 °C, 20 min) (Figure 3). However, the activity of water-soluble fraction was lost by passing through the dialysis membrane of the molecular weight cut off 1000 (Figure 4). These results suggested that the active agent in Kefram-Kefir was stable and relatively small molecule. The water-soluble fraction was applied to Bio-Gel P-2 column to refine the molecular size of the active agent (Figure 5a). It is resulted that fraction 3 highly augmented 2-deoxyglucose uptake activity of L6 myotubes (Figure 5b). Since phenol red (molecular weight: 354.4)

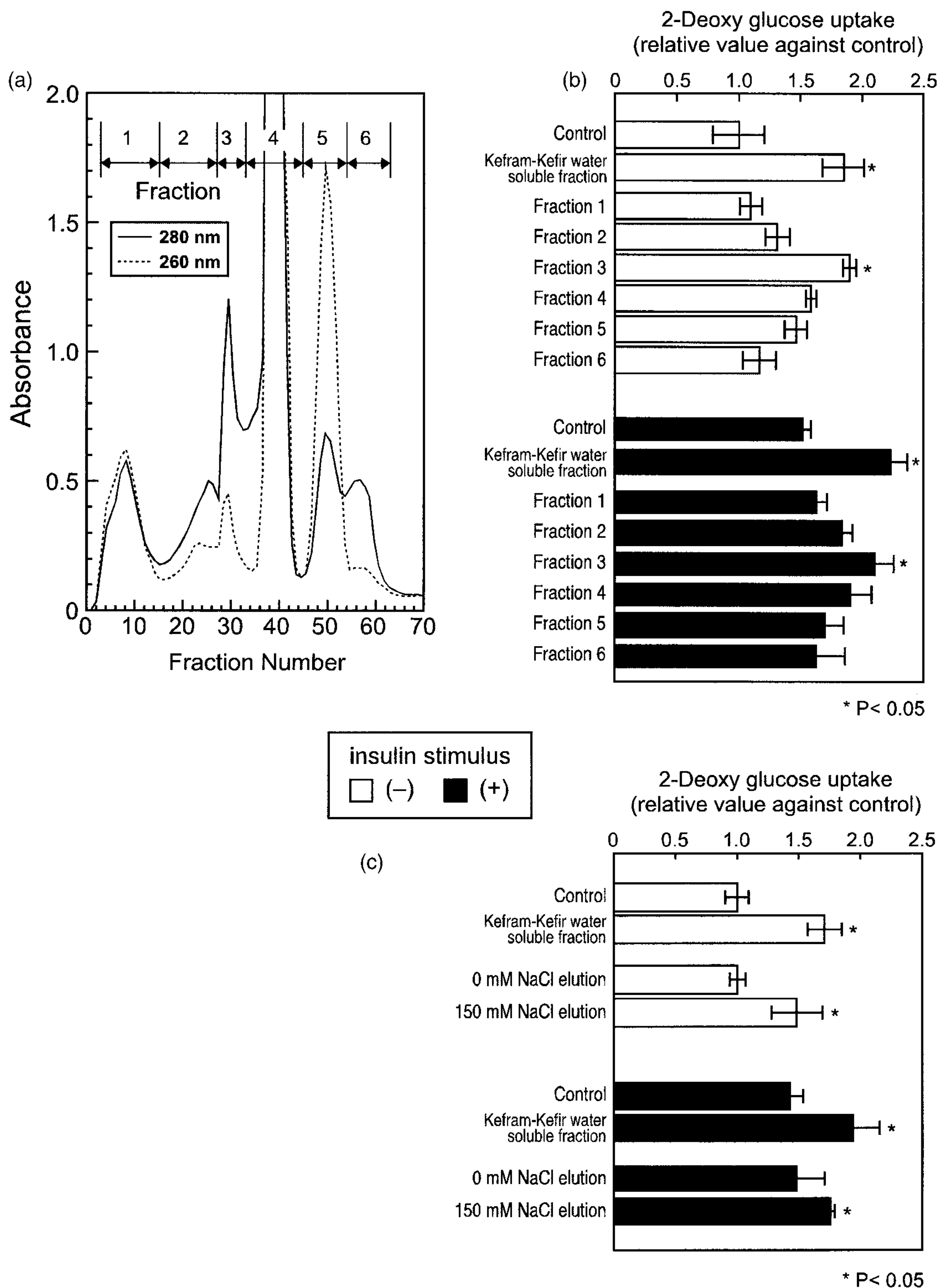


Figure 5. Partial purification of the active agent in Kefram-Kefir. (a) Gel filtration profile of the water-soluble fraction on Bio-Gel P-2 column. (b) Enhancement of glucose uptake into L6 myotubes by each fraction. (c) Negative charged property of the active agent included in fraction 3. L6 myotubes were cultured with the media supplemented with 25% (v/v) of each samples for 4 h, then the glucose transport determination was performed. Significant difference ($P < 0.05$) compared with the control.

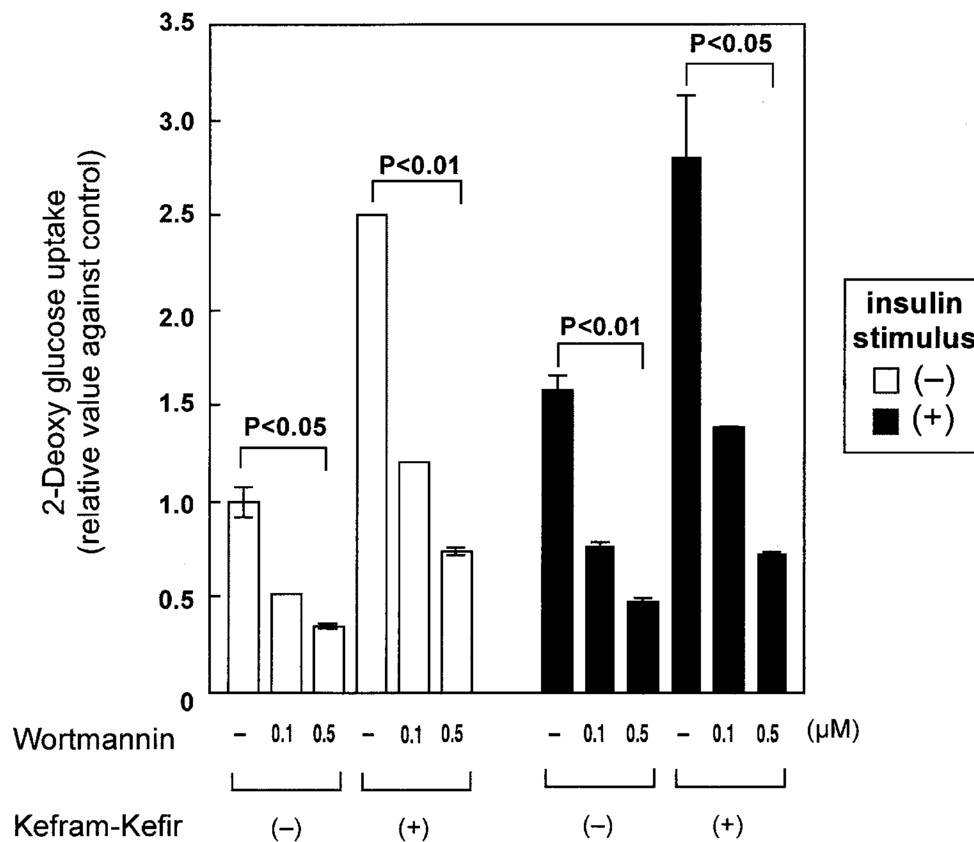


Figure 6. Effects of PI 3-kinase inhibition on glucose uptake into L6 myotubes with the water-soluble fraction of Kefram-Kefir. The L6 myotubes were treated with 0.1 or 0.5 μM of wortmannin for 24 h at 37 °C, the cells were treated with the water-soluble fraction of Kefram-Kefir in the presence of wortmannin for 4 h, and the glucose transport determination was performed. Significant difference ($P < 0.01$ or 0.05) compared with the cells treatment without wortmannin.

was eluted out around the center part of the fraction 4 on the same column (data not shown), the molecular weight of the active agent was estimated to about 500. Furthermore, fraction 3 applied to anion-exchange TSK-gel DEAE-Toyopearl 650M column was resulted in the active agent eluted by 150 mM NaCl (Figure 5c). This suggested that the active agent was anionic. Furthermore, this augmentation was almost inhibited in the presence of the PI 3-kinase inhibitor wortmannin (Figure 6). However, the inhibition of glucose uptake by wortmannin was not completely. It was suggested that the other pathway of independent PI 3-kinase in glucose uptake enhancing by Kefram-Kefir. Therefore, it was suggested that glucose uptake enhancement of Kefram-Kefir was associated with activation of a typical insulin signaling pathway and the other pathway.

Discussion

Insulin stimulates uptake of blood glucose into a target organ such as the muscle, the adipose or the liver. Disruption of this mechanism causes hyperglycemia, which is a type of diabetes mellitus. Recently, it was reported that ROS played an important role in the two types of diabetes mellitus, IDDM and NIDDM (Rudich et al., 1997, 1998). It was indicated that the insulin resistance of the target organ in NIDDM patients was a dysfunction of insulin signal transduction, especially PI 3-kinase as a key enzyme in it. Since the ROS influenced the insulin signal transduction into the inactivated state, many approaches using anti-oxidative agents were investigated. The traditional fermented milk, Kefram-Kefir, showed valuable effects such as anti-cancer effects, enhancing of the immune system and also acting as an anti-oxidant. Therefore, we considered that Kefram-Kefir to be ef-

fective for diabetes mellitus. Then we evaluated the value of Kefram-Kefir on glucose uptake in skeletal muscle cells specifically *in vitro*.

The water-soluble fraction of Kefram-Kefir showed excellent activity to enhance glucose transport on the L6 skeletal muscle cells. The active agent in the fraction was extremely stable in the various conditions such as heating or pH change. In addition, the active agent was estimated to be a small molecule. It was considered that these excellent properties were very useful for therapeutic use. For example, it was suggested that the active agent which was oral-administrated to NIDDM patients was resistant to gastric juices or pH change in the duodenum, easily absorbed in the intestine by its small molecular size, and augmented the glucose uptake in the insulin-target organs.

It was reported that PI 3-kinase located in the key point of the insulin signaling pathway might participate in the translocation of glucose transporter GLUT4 to the cell membrane (Vlahos et al., 1994; Tanishita et al., 1997). The treatment of the PI 3-kinase inhibitor wortmannin led to a decrease in the glucose uptake activity of the water-soluble fraction of Kefram-Kefir. It was suggested that the water-soluble fraction of Kefram-Kefir activated the PI 3-kinase or other upstream molecules in the insulin signaling pathway, resulting in the augmented glucose uptake. However, it was not observed completely inhibition of glucose uptake by wortmannin. This suggested that Kefram-Kefir activated the other PI 3-kinase independent pathway. What is the active agent in Kefram-Kefir? The answer will greatly contribute to the therapy of diabetes mellitus and also the investigation for insulin signal transduction.

In conclusion, fermented milk, Kefram-Kefir augmented glucose uptake in L6 myotubes and the results which suggested the possibility of therapeutic use for type 2 diabetes mellitus. The active agent included in water-soluble fraction of Kefram-Kefir is stable at a high temperature and pH 4-10, small molecular in weight and anion charged. It is suggested two pathways of the glucose uptake augmentation by the water-soluble fraction of Kefram-Kefir from the treatment of wortmannin; (1) the active agent in Kefram-Kefir mainly augments glucose uptake via the insulin signaling pathway including PI 3-kinase and activates PI 3-kinase or other upstream molecules in the insulin signaling pathway, (2) the active agent in Kefram-Kefir also activates the PI 3-kinase independent pathway.

We believe that identification and characterization of the active agent included in Kefram-Kefir and further investigation of molecular mechanisms will provide new insights into the effective therapeutic approaches for type-2 diabetes mellitus.

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