Biochemical and Biophysical Research Communications 445 (2014) 170–174

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

# Lowered extracellular pH is involved in the pathogenesis of skeletal muscle insulin resistance

Hiroki Hayata [a,1], Hiroaki Miyazaki [a,c,1,*], Naomi Niisato [a,c], Noriko Yokoyama [a], Yoshinori Marunaka [a,b,c,*]

[a] Department of Molecular Cell Physiology, Graduate School of Medical Science, Kyoto Prefectural University of Medicine, Kyoto, Japan
[b] Department of Bio-Ionomics, Graduate School of Medical Science, Kyoto Prefectural University of Medicine, Kyoto, Japan
[c] Japan Institute for Food Education and Health, Heian Jogakuin (St. Agnes') University, Kyoto, Japan

ARTICLE INFO

*Article history:*
Received 30 December 2013
Available online 3 February 2014

*Keywords:*
Insulin
pH
Receptor
Skeletal muscle

ABSTRACT

Insulin resistance in the skeletal muscle is manifested by diminished insulin-stimulated glucose uptake and is a core factor in the pathogenesis of type 2 diabetes mellitus (DM), but the mechanism causing insulin resistance is still unknown. Our recent study has shown that pH of interstitial fluids was lowered in early developmental stage of insulin resistance in OLETF rats, a model of type 2 DM. Therefore, in the present study, we confirmed effects of the extracellular pH on the insulin signaling pathway in a rat skeletal muscle-derived cell line, L6 cell. The phosphorylation level (activation) of the insulin receptor was significantly diminished in low pH media. The phosphorylation level of Akt, which is a downstream target of the insulin signaling pathway, also decreased in low pH media. Moreover, the insulin binding to its receptor was reduced by lowering extracellular pH, while the expression of insulin receptors on the plasma membrane was not affected by the extracellular pH. Finally, insulin-stimulated 2-deoxyglucose uptake in L6 cells was diminished in low pH media. Our present study suggests that lowered extracellular pH conditions may produce the pathogenesis of insulin resistance in skeletal muscle cells.



## 1. Introduction

Insulin stimulates glucose uptake in skeletal muscles, especially when blood glucose level rises after a meal. In the pathogenesis of insulin resistance, insulin insufficiently stimulates glucose uptake in skeletal muscles [1], resulting in sustained high levels of blood glucose after meals, which is a typical symptom of type 2 diabetes mellitus (DM). Type 2 DM is complicated with hypertension, dyslipidemia, and micro- and macro-vascular diseases. The number of patients with diabetes is still increasing, and this disease is becoming a worldwide social problem. Therefore, understanding of the mechanisms underlying insulin resistance is important for overcoming diabetes mellitus. However, the mechanisms of insulin resistance are still unclear.

Insulin-stimulated glucose uptake in skeletal muscle cells is mainly mediated by glucose transporter 4 (GLUT4), which plays a key role in whole-body glucose homeostasis [2]. The main action of insulin is the regulation of cellular glucose uptake into skeletal muscle cells and adipocytes via GLUT4 translocation from intracellular store sites to the plasma membrane. This dynamic process is retained through a continuous recycling and relocation of GLUT4 between the plasma membrane and intracellular compartments. The regulatory mechanisms of intracellular recycling of GLUT4 are well identified [3]. Insulin stimulates glucose uptake in skeletal muscle mainly in a phosphatidylinositol 3-kinase (PI3K)-dependent pathway after binding to insulin receptor on the plasma membrane. Insulin binding to its receptor on the plasma membrane immediately leads to auto-phosphorylation on tyrosine residues of the receptor. Subsequently, insulin receptor substrate-1 (IRS-1) is auto-phosphorylated on its tyrosine residues. The phosphorylated IRS-1 in turn leads PI3K to the active form. Active PI3K then catalyzes 3′ phosphorylation of phosphatidylinositol 4,5-diphosphate, which leads to activation of Akt. All components of the PI3-Kinase/Akt-mediated signal pathway take a part in the intracellular translocation of GLUT4. Defects in insulin signal transduction through these pathways are associated with reduced activity of insulin-stimulated glucose transport in skeletal muscles of type 2 diabetic patients [4]. However, the primary mechanism causing insulin resistance leading to type 2 DM has not yet been elucidated.

* Corresponding authors at: Department of Molecular Cell Physiology, Graduate School of Medical Science, Kyoto Prefectural University of Medicine, Kyoto 602-8566, Japan. Fax: +81 75 251 0295.
*E-mail addresses:* hmiyazak@koto.kpu-m.ac.jp (H. Miyazaki), marunaka@koto.kpu-m.ac.jp (Y. Marunaka).
[1] These authors contributed equally to the present study.

http://dx.doi.org/10.1016/j.bbrc.2014.01.162
0006-291X/

Our recent study has shown that pH of interstitial fluids is low in Otsuka Long-Evans Tokushima Fatty (OLETF) rats, a model of type 2 DM [5]. Many epidemiological studies have recently reported the relationship between metabolic acidosis and insulin resistance [6]. However, it is still unclear if extracellular pH affects insulin action in skeletal muscle cells. To further investigate the role of interstitial fluid pH in the mechanism causing insulin resistance, we analyzed the effect of lowered extracellular pH on insulin action in the rat skeletal muscle L6 cells.

## 2. Materials and methods

### 2.1. Cell culture and differentiation

L6 myoblasts, a cell line derived from rat skeletal muscle, were obtained from JCRB Cell Bank (Osaka, Japan). L6 myoblasts were grown in Dulbecco's modified Eagle medium (DMEM) (Sigma–Aldrich, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS), 100 μg/ml streptomycin and 100 U/ml penicillin (Wako Pure Chemical, Osaka, Japan) in a humidified incubator at 37 °C with 5% $CO_2$ in culture dishes or 24 well plates. After reaching 70–80% confluence, the L6 myoblasts were differentiated to myotubes by culture with DMEM containing 2% FBS. Subsequently, the cells were cultured for 7–8 days (for Western blotting and binding assay) or 12 days (for 2-deoxyglucose uptake) under this condition.

### 2.2. Western blotting

Following serum starvation for 4 h, differentiated L6 myotubes were incubated in HEPES buffer (135 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 5.5 mM glucose, and 10 mM HEPES) under different pH conditions (7.4, 7.2, 7.0, and 6.8) with 100 nM insulin at 37 °C for 15 min. Then, the cells were lysed with ice-cold lysis buffer (150 mM NaCl, 1.5 mM $MgCl_2$, 50 mM HEPES, 1 mM EGTA, 10% Glycerol, 1% Triton X-100, 100 mM NaF, 10 mM pyrophosphate, 100 μg/ml aprotinin, 250 μg/ml leupeptin, 1 mM PMSF, and 200 μM Na-orthovanadate). The samples were separated on SDS–polyacrylamide gel electrophoresis (SDS–PAGE), and proteins were transferred to nitrocellulose membranes. The blots were incubated with primary antibodies (anti-insulin receptor β subunit, anti-phospho-insulin receptor β subunit (Tyr 1146), anti-Akt, anti-phospho-Akt (Ser 473), and anti-phospho-Akt (Thr 308) obtained from Cell Signaling Technology (Beverly, MA, USA), and then were detected by ECL prime (GE healthcare, Buckinghamshire, UK). The band densities were measured with ImageJ (National Institutes of Health, Bethesda, MD, USA). The content of each phosphorylated protein was normalized by that of total proteins.

### 2.3. Cell surface biotinylation of insulin receptor

After serum starvation for 4 h, differentiated L6 myotubes were treated with 100 nM insulin for 15 min in the HEPES buffers at different pH values (7.4, 7.2, 7.0, and 6.8). Then, cells were washed with ice-cold HEPES buffer (pH 7.4), and were incubated with 0.5 mg/ml EZ-Link Sulfo-NHS-biotin (Thermo Scientific), a membrane-impermeable biotinylation reagent, in HEPES buffer for 20 min at 4 °C. After stopping the biotinylation reaction by incubating with 50 mM glycine in HEPES buffer for 5 min at 4 °C, cellular extracts were prepared as described above. The cellular lysates were diluted to 200 μl (containing 300 μg protein) with NeutrAvidin Agarose Resins (Thermo Scientific), and then incubated overnight at 4 °C with rotating. NeutrAvidin-precipitated complexes were rinsed three times with lysis buffer and biotinylated proteins were eluted with 2 × SDS sample buffer (0.5 M Tris, 20% glycerol, 4% sodium dodecyl sulfate, 10% 2β-mercaptoethanol, and pH 6.8 titrated with HCl) by boiling at 65 °C for 10 min. Protein samples were separated by SDS–PAGE, and cell surface expressed and total insulin receptor β subunits were respectively processed for Western blotting (as described above). Blots were stripped, and were reblotted with anti-$Na^+$, $K^+$-ATPase antibody (Millipore, Billerica, MA, USA) as a positive control for expression on the plasma membrane, and with anti-GAPDH antibody (Cell Signaling Technology) as a negative control for expression on the plasma membrane.

### 2.4. Insulin binding assay

Insulin binding to its receptor and expression of insulin receptor were estimated with the method reported by Wilson et al. [7]. In brief, L6 cells were seeded on 24 well plates, and were differentiated to myotubes. The differentiated myotubes were incubated in HEPES buffers with different pH values (pH 7.4, 7.2, 7.0, and 6.8) containing [$^{125}$I]-labeled insulin (~100,000 cpm/ml) (MP Biomedicals, Santa Ana, CA, USA) for 15 min at room temperature. After 15 min incubation, the incubation medium was removed. The cells were washed twice with HEPES buffer, and were suspended by adding EDTA to the buffer. The radioactivity of cell suspension was measured in a γ-scintillation counter (Packard Cobra Quantum 5002, PerkinElmer, Waltham, MA, USA). Non-specific [$^{125}$I]-labeled insulin binding was determined by obtain the specific insulin binding in the presence of an excess (1 μM) of unlabeled insulin.

### 2.5. 2-Deoxyglucose (2DG) uptake measurement

L6 cells were seeded on 24 well plates, and were differentiated to myotubes. The differentiated cells were serum starved for 4 h, and were incubated with Krebs-Ringer-HEPES (KRH) buffer (137 mM NaCl, 4.7 mM KCl, 1.85 mM $CaCl_2$, 1.3 mM $MgSO_4$, 10 mM HEPES, and 100 nM insulin), whose pH was adjusted to 7.4, 7.2, 7.0, and 6.8 with NaOH for 15 min at 37 °C in air. Then, 6.5 mM 2DG containing 0.17 μCi/ml [$^3$H]-2DG was added to KRH buffer, and cells were incubated for 5 min. 2DG uptake was terminated by ice-cold KRH buffer. Cells were lysed with 0.05 N NaOH. Radioactivity was measured by Tri-Carb2810TR liquid scintillation counter (PerkinElmer). Nonspecific 2DG uptake was estimated by adding 20 μM cytochalasin B, an inhibitor of GLUT4 participating in the insulin-stimulated glucose transport. The protein concentration of each well was determined by BCA assay kit (Thermo Scientific, Waltham, MA, USA). 2DG uptake in L6 cells was normalized by protein content in each well.

### 2.6. Statistical analysis

Data are expressed as means ± SEM. Differences were appropriately analyzed by Student's *t*-test or Dunnett's multiple comparison test, and were considered significant at the level of $p < 0.05$.

## 3. Results

### 3.1. The phosphorylation level of insulin receptor is diminished in low pH media

Insulin binding to its receptor results in receptor phosphorylation on their tyrosine residues; three tyrosine residues (Tyr1146, Tyr1150 and Tyr1151) within the kinase domain of insulin receptor β subunit are major auto-phosphorylation sites, and the phosphorylation is necessary for kinase activation [8]. Therefore, we examined the effect of extracellular pH on the phosphorylation level of Tyr 1146 of insulin receptor after stimulation of 100 nM insulin using Western blotting. As shown in Fig. 1, the

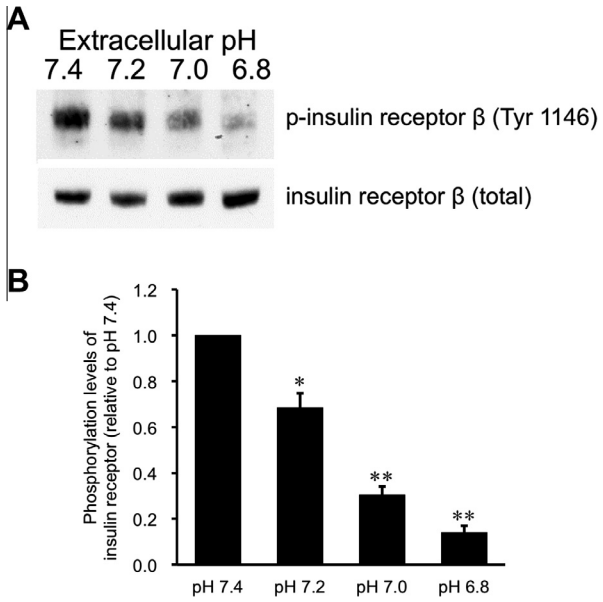


**Fig. 1.** Phosphorylation levels of insulin receptor. After serum starvation for 4 h, L6 myotubes were treated with 100 nM insulin for 15 min in the buffer with different pH. Total cell lysates were isolated and analyzed by Western blotting with indicated antibodies. (A) Representative blots are shown. (B) The quantitative values of expression of insulin receptor using densitometry from 6 independent experiments using anti-phospho-insulin receptor-β (Tyr 1146) normalized to the level of total insulin receptor compared with that in pH 7.4 buffer. The values are shown as means ± SEM ($n = 6$). $^{*}p < 0.05$, $^{**}p < 0.01$ vs pH 7.4.

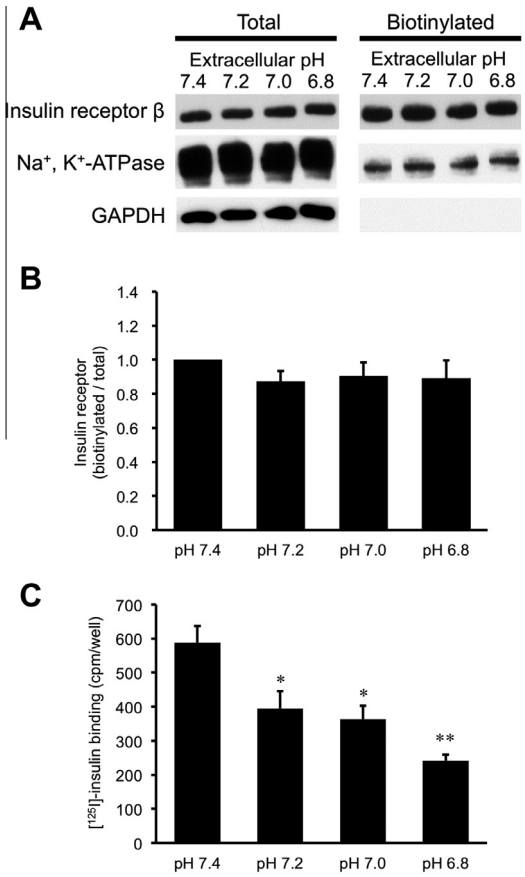


**Fig. 2.** Effects of extracellular pH on the expression of insulin receptor on the plasma membrane and the insulin binding to insulin receptor. After serum starvation for 4 h, L6 myotubes were treated with 100 nM insulin for 15 min in the HEPES buffer with different pH. Proteins expressed on the plasma membrane were biotinylated and precipitated. (A) Representative blots of total expression of insulin receptor on the plasma membrane, the $Na^+$, $K^+$-ATPase, and GAPDH. (B) Quantitative data of expression of insulin receptor on the plasma membrane at different pH normalized to that at pH 7.4. The results are presented as means ± SEM ($n = 8$). There was no statistically significant difference. (C) Differentiated L6 myotubes were treated with [$^{125}$I]-labeled insulin for 15 min in the indicated pH buffers, and the radioactivities were measured after cells were washed and suspended. The values of radioactivity from at least 6 experiments are shown. The values are shown as means ± SEM are shown. $^{*}p < 0.05$, $^{**}p < 0.01$ vs pH 7.4.

phosphorylation level of insulin receptor was significantly diminished in the media whose pH was 7.2 or less than 7.2. However, the total expression level of insulin receptor was not changed under each pH condition (Fig. 1A). When L6 cells were exposed to media without insulin, the basal phosphorylation of insulin receptor was not detectable under our experimental condition (data not shown). These results suggest that low extracellular pH diminishes insulin-induced activation of insulin receptor.

### *3.2. Expression of insulin receptors on the plasma membrane is not changed in low pH media*

As described above, the auto-phosphorylation level of insulin receptor in L6 cells was diminished in low pH media. As the basis of this result, we considered a possibility that the number of insulin receptors expressed on the plasma membrane was diminished by treatment with low pH media. To investigate this possibility, the expression of insulin receptors on the plasma membrane was confirmed by the cell-surface biotinylation method under different pH conditions. As shown in Fig. 2A and B, the expression level of biotinylated insulin receptors was not changed by lowering pH (7.2, 7.0, and 6.8) media compared with that in normal pH (7.4) media. These results suggest that the reduction of extracellular pH does not affect the expression of insulin receptor on the plasma membrane.

### *3.3. The insulin binding to insulin receptor is reduced in low pH media*

The expression of insulin receptor on the plasma membrane in L6 cells was not affected by the reduction of extracellular pH; nevertheless, insulin-stimulated phosphorylation levels of insulin receptors were significantly diminished by acidification of extracellular media. Therefore, we hypothesized a possibility that lowering extracellular pH diminishes the insulin binding to its receptor, attenuating phosphorylation of insulin receptor. To confirm this possibility, we examined the insulin binding to its receptor with binding assay using radiolabeled insulin. In L6 cells, the insulin binding to insulin receptor was significantly suppressed at lower pH conditions (Fig. 2C). From these results, the impaired auto-phosphorylation of insulin receptor in low pH media is caused by lowered ligand binding to the receptor.

### *3.4. The phosphorylation level of Akt is diminished in low pH media*

Akt is a downstream target in the insulin signaling pathway including stimulation of glucose uptake in skeletal muscle cells [2]. Therefore, we next investigated the phosphorylation level of Akt. While Akt has several phosphorylation sites, residues Ser473 and Thr308 are recognized to be essentially important for activation of Akt [9]. We therefore examined phosphorylation levels of these residues of Akt. As shown in Fig. 3, the phosphorylation level of each residue in Akt significantly decreased under lower pH conditions. Without insulin in the media (under the basal condition), the phosphorylation level of Akt was very low (not detectable), and it was impossible to compare the insulin-stimulated phosphorylation level with the basal one (data not shown). These

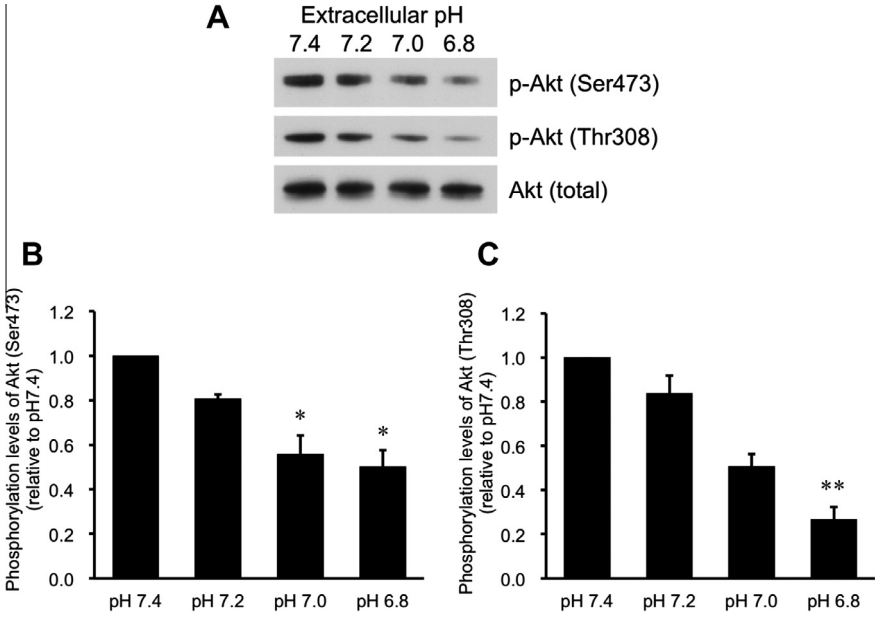


**Fig. 3.** Phosphorylation levels of Akt. L6 myotubes were treated with 100 nM insulin for 15 min in the buffer with different pH after serum starvation for 4 h. Total cell lysates were isolated, and were analyzed by Western blot with the indicated antibodies. (A) Representative blots are shown using anti-phospho-Akt. Phosphorylation levels of Ser473 (B) and Thr308 (C) are expressed as normalized values to the level of total Akt compared with those in pH 7.4. The values are shown as mean ± SEM ($n = 6$). $^{*}p < 0.05$, $^{**}p < 0.01$ vs pH 7.4.

results suggest that the lower extracellular pH disturbs insulin-induced activation of Akt.

### 3.5. The 2-deoxyglucose (2DG) uptake is diminished in low pH media

Insulin decreases the blood glucose level mainly by increasing glucose uptake into skeletal muscle cells. The decrease in insulin-stimulated glucose uptake is thought to be one of the main factors of insulin resistance in skeletal muscle cells [10]. Therefore, we examined the effect of extracellular pH on glucose uptake by using radiolabeled 2DG, a glucose analogue, which is transported into cells in the same manner as glucose, and hexokinase phosphorylates 2DG converting to deoxyglucose-6-phosphate, which is not further metabolized accumulating in the cells. As shown in Fig. 4, insulin increased 2DG uptake in L6 cells under all tested pH conditions. However, the insulin-stimulated 2DG uptake was significantly suppressed when the cells were stimulated with insulin in the pH 6.8 media. Though there were no statistically significant differences, the insulin-stimulated 2DG uptake showed a tendency to be lower in pH 7.2 and 7.0 media than that in pH 7.4 medium (Fig. 4). These data suggest that insulin could not fully stimulate the glucose uptake by skeletal muscle cells under lower extracellular pH conditions.

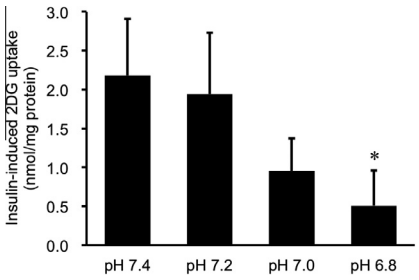


**Fig. 4.** Insulin-stimulated 2-deoxyglucose (2DG) uptake. After serum starvation for 4 h, L6 myotubes were exposed to 100 nM insulin for 15 min in the different pH buffers, and then were added with 6.5 mM 2-deoxyglucose (DG) containing [$^3$H]-labeled 2-DG for 5 min. The radioactivities were measured after cells were washed and lysed with NaOH. Nonspecific uptake was determined by adding cytochalasin B, GLUT inhibitor, simultaneously. Values of 2DG taken up into the cell were determined from the radioactivity, and were normalized to protein concentration by BCA assay. The values are shown as means ± SEM ($n = 6$). $^{*}p < 0.05$ vs pH 7.4.

## 4. Discussion

In the present study, we revealed that the following observations in L6 cells: (1) the insulin-stimulated phosphorylation of insulin receptor was significantly lower under the condition of lower extracellular pH than that under normal pH (7.4) medium, (2) the insulin binding to insulin receptor was down-regulated in lowered pH (7.2, 7.0 and 6.8) media, and (3) phosphorylation of Akt, a downstream regulator of insulin signaling cascades for glucose uptakes, and uptake of 2-deoxy-D-glucose (2DG; non-metabolizable glucose analog) were reduced in the low pH (6.8) medium.

In the present study, we found that the insulin-stimulated phosphorylation level of insulin receptor was significantly diminished under lower extracellular pH conditions compared with that under normal pH condition (Fig. 1). The insulin receptor is a heterotetrameric bifunctional complex, consisting of two extracellular α subunits bound to insulin and two transmembrane β subunits containing tyrosine kinase activity. Insulin binding to the α subunit induces auto-phosphorylation of β subunit immediately after insulin binds to its receptor, resulting in increased down-stream signaling activity of the receptor [11,12]. The activated insulin receptor then phosphorylates intracellular substrates, which are involved in the activation of glucose transport. Therefore, we speculated that the declined level of phosphorylation of insulin receptor in low pH media could be caused by lowered insulin binding to insulin receptor. Indeed, we found that the binding of [$^{125}$I]-insulin to L6 cells significantly decreased in lower pH conditions compared to the normal pH (7.4). Under a condition with pH 6.8, the [$^{125}$I]-insulin binding to the L6 cells was decreased by up to 50% of control (Fig. 2C). Some investigators have suggested that a reduced insulin action under acidic conditions might be due to diminution of insulin binding to its receptor in fibroblasts, adipocytes, and

hepatocytes [13–16]. However, little information is available on the relationship between the extracellular pH and insulin action in muscles [17,18], one of the main targets of insulin-stimulated glucose uptake leading to reduction of blood glucose level. Further, it is notable that these investigators [13–16] have used experimental protocols based on the acidosis (*i.e.*, low pH of blood) but not the pH of extracellular fluids (interstitial fluids). The insulin receptor faces the interstitial fluid but not directly blood, however it is not yet known if the pH of extracellular fluids (interstitial fluids) is really low in diabetic condition. Our studies [5,19] have clearly indicated that the pH of interstitial fluids, which insulin receptors located on the plasma membrane of cells directly face, is lower in diabetic rats than that in non-diabetic rats. Taken together with these studies [5,19] and the present study, we indicate that lowered pH of interstitial fluids under diabetic conditions diminishes the insulin biding affinity to its receptor.

We also revealed that the phosphorylation level of Akt, a downstream target of the insulin receptor-signaling cascade was also reduced in the low pH condition (Fig. 3). GLUT4, a key molecule for insulin-regulated glucose uptake, is located in intracellular store sites under the non-stimulated condition, however it is translocated to the plasma membrane when cells are stimulated by insulin [3]. Activation of Akt is an important factor for translocation of GLUT4 to the plasma membrane following insulin stimulation [3]. As shown in Fig. 3, the phosphorylation level of Akt was decreased in low pH media. This phenomenon would lead to the lowered number of GLUT4 located on the plasma membrane under low pH media, resulting in diminution of insulin-stimulated 2DG uptake mediated by GLUT4 located on the plasma membrane.

We should consider a possibility that lowered extracellular pH would diminish the insulin-stimulated phosphorylation levels of insulin receptor and Akt via a decrease in intracellular pH, which may in turn affect kinase activities of cytosolic proteins, although we indicate that lowered pH of interstitial fluids diminished the insulin binding to its receptor. However, as shown in Figs. 1 and 3, the pattern of the declined phosphorylation level of Akt was very similar to that of insulin receptor, meaning that it is likely that the decreased phosphorylation level of insulin receptor directly influences the phosphorylation level of Akt and the effect of intracellular pH change would be relatively a small factor for the phosphorylation. Although further studies are still required to evaluate effects of intracellular pH on insulin signaling, we consider that lowered phosphorylation levels of proteins in the insulin signaling pathway are likely to be due to the lowered insulin-binding to insulin receptor caused by lower pH conditions.

In general, body fluids of diabetes patients would be acidic mainly due to elevation of ketone body production. In addition, an elevation of lactic acid production in metabolic tissues would be also likely involved in the body fluid acidosis. The organic acids-induced acidosis could contribute to the development of insulin resistance. Several studies [20–22] have suggested a close correlation between organic acid production and insulin sensitivity in both type 2 DM patients and healthy subjects. Recent epidemiological studies have confirmed that the relationship between insulin resistance and metabolic acidosis-caused phenomena, such as low serum bicarbonate and low urine pH [6]. Moreover, our recent study has demonstrated that lowered interstitial fluid pH in a diabetes mellitus model rat, Otsuka Long-Evans Tokushima Fatty (OLETF) rat [5], is recovered by feeding propolis (a resinous mixture collected from plants by bees) diet that improves insulin sensitivity and blood pressure [5]. Therefore, it is probable that lowered pH is closely related with insulin resistance. In the present study, we indicate that the effect of insulin is more insufficient under the condition of lowered pH compared with normal pH, and so the low pH condition in interstitial fluid would cause insulin resistance, the main pathogenesis of type 2 DM.

In conclusion, the pH of interstitial fluids is the key factor causing pathogenesis of insulin resistance in type 2 DM by acting as a regulator controlling the insulin sensitivity.

## Acknowledgments

This study was supported by Grants-in-Aid in part from Japan Society of the Promotion of Science (24590283, 25460297, 25670111), The Salt Science Research Foundation (1235), and Research Conference for Cell Function.

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