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Glyceraldehyde-3-phosphate dehydrogenase interacts with phosphorylated Akt resulting from increased blood glucose in rat cardiac muscle

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ABSTRACT

Here we describe the interaction of phosphorylated ~40 kDa protein with phosphorylated Akt which is a serine/threonine kinase resulting from increased blood glucose in rat cardiac muscle. Mass spectrometry analysis revealed that this protein was glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Furthermore, increase in Akt and GAPDH phosporylation and induction of their association were both observed after insulin stimulation in the H9c2 cell line derived from embryonic rat ventricle. Moreover, the activation of GAPDH was upregulated when the GAPDH phosphorylation was increased. Our data suggest that GAPDH phosphorylation and association with Akt by insulin treatment have some bearing on the enhancement of GAPDH activity.

Structured summary:
MINT-7891324, MINT-7891304, MINT-7891314: *GAPDH* (uniprotkb:P04797) *physically interacts* (MI:0915) with *Akt* (uniprotkb:P47196) by *anti bait coimmunoprecipitation* (MI:0006)

1. Introduction

Akt, which is a serine/threonine kinase, has a central role in the regulation of several signaling pathways controlling cell survival and proliferation [1]. This kinase has two distinct functional domains: an N-terminal PH domain mediating protein-protein and protein-lipid interactions and a C-terminal catalytic domain. The interaction of PIP3 with the PH domain of Akt promotes the translocation of Akt to the plasma membrane, where it undergoes phosphorylation at two sites; one in the activation loop (Thr308) (by phosphoinositide-dependent protein kinase 1 (PDK1)) and the other in the carboxy-terminal (C-terminal) tail (Ser473) (by PDK2) [1–3].

Insulin resistance and altered glucose and lipid metabolism in multiple organs are defined as subnormal biologic responses to a given concentration of insulin and are characteristic of many disease states, including type 2 diabetes, obesity and normal aging process, which collectively constitute risk factors for the development of atherosclerotic cardiovascular disease [4–6]. The mechanism underlying insulin resistance involves deleterious effects on insulin signaling and glucose transport activity [7–9]. Insulin-induced Akt phosphorylation was decreased in muscle and liver of the animal model of insulin resistance, streptozotocin (STZ)-treated rats, compared to normal rats [10].

To date, detailed studies on insulin signaling have accumulated significant information. However, the full identification of the molecular signaling events involved in insulin actions is still in progress.

To investigate the downstream cascade of Akt in cardiac muscle, we used rat cardiac muscle after glucose intraperitoneal injection. The phosphorylation of Akt was enhanced and phosphorylated ~40 kDa protein (p40) interacted with Akt after glucose injection in cardiac muscle. Peptide mass mapping of this protein revealed that the protein is glyceraldehyde-3-phosphate dehydrogenase (GAPDH). GAPDH interacted with Akt after insulin stimulation was also phosphorylated and its activity was upregulated. These results provide direct evidence for the first time linking Akt to GAPDH.

Abbreviations: EDL, extensor digitorum longus; IP, immunoprecipitate; MALDI-TOF, matrix-assisted laser desorption/ionization time-of-flight; PVDF, polyvinylidene difluoride; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PDK, phosphoinositide-dependent protein kinase

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2. Materials and methods

2.1. Materials

Antibodies used for western blotting were: protein G sepharose (GE healthcare); anti-GAPDH (Santa Cruz Biotechnology); anti-phospho Ser/Thr/Tyr (Abcom); anti-phospho Akt (ser473); anti-Akt (Cell Signaling Technology); anti-mouse IgG alkaline phosphatase conjugate (Sigma); anti-rabbit IgG alkaline phosphatase conjugate (Promega).

2.2. Animals

Animal experiments were performed in accordance with Juntendo University guidelines. Wistar rats weighing 480–510 g were obtained from Japan SLC, Inc. (Shizuoka, Japan). They were housed on a 12 h light–12 h dark photoperiod (lights off 09:00–21:00) in an environmentally controlled room (23 ± 1 °C, 55 ± 5% relative humidity), and received standard rat chow and water ad libitum.

2.3. Muscle removal

Rats were fasted overnight (15–18 h) before muscle isolation. Rats were then anesthetized with 50 mg/kg sodium pentobarbital administered intraperitoneally. After a surgical plane of anesthesia was reached, glucose (1 g/kg body weight) was administered by intraperitoneal injection to the animals. Blood samples were taken and several muscles (cardiac muscle, soleus, and extensor digitorum longus (EDL)) were removed quickly at 30, 60, and 120 min after glucose injection or no glucose injection. Glucose concentrations were measured with a commercial test kit (Glutest Ace R, Sanwa Kagaku, Japan) at every time point.

2.4. Cell line, cell culture, cell stimulation

H9c2 cell line derived from embryonic rat ventricle was provided by T. Okada (Juntendo University, Tokyo, Japan). This cell line was cultured in DMEM (Invitrogen) with 10% FBS (Biowest), and penicillin/streptomycin at 37 °C in a humidified atmosphere. Cells (1×10^7) were suspended in PBS and treated with insulin (Sigma) at 37 °C.

2.5. Sample preparations

Muscles and cells were homogenized in ice-cold buffer containing 10 mM Tris-HCl (pH 7.7), 150 mM NaCl, 1% Nonidet P-40, with PhosSTOP (Roche) and Complete (Roche). Lysates were centrifuged for 20 min at 4 °C to remove insoluble materials.

2.6. Immunoprecipitation and immunoblot analysis

Immunoprecipitation and immunoblot experiments were conducted as previously described [11]. Briefly, lysates were immunoprecipitated with the appropriate Abs. Proteins were separated by 10% SDS–PAGE and transferred to polyvinylidene difluoride (PVDF) membrane. The membranes were incubated with the appropriate Abs. The blots were visualized by incubation with alkaline phosphatase-labeled secondary antibody and then developed by Immun-Star™AP substrate (Bio-Rad Laboratories).

2.7. Identification of proteins by peptide mass mapping

Cardiac muscle lysates were immunoprecipitated with anti-Akt antibody and protein G-Sepharose. The sample was separated by 10% SDS–PAGE and transferred to PVDF membrane. Then this membrane was stained with Coomassie brilliant blue. The PVDF-immobilized proteins were digested in situ with trypsin. Molecular mass analyses of trypsin fragments were performed by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry using an ABI Per-Septive Biosystem Voyager DE/RP (Applied Biosystems). Identification of proteins was conducted by comparison between the molecular mass determined by MALDI-TOF/MS and theoretical peptide masses from the proteins registered in ExPASy.

2.8. Assay for GAPDH activity

The activity of GAPDH was measured spectrophotometrically according to the methods of Stech and Kant [12] with some modification. Cell lysates were incubated in 100 mM TAPS (pH 8.5), 1 mM EDTA (pH 8.0), 1 mM NAD^+, 15 mM Na_3AsO_4 (pH 8.0) and 10 mM dithiothreitol at 37 °C for 5 min to establish a baseline. The reaction was initiated by addition of 1 mM gryceraldehyde-3-phosphate, and absorbance at 340 nm was measured for 5 min at 37 °C. Activity is expressed as the concentration (μM) of NADH generated during the assay.

3. Results

3.1. Phosphorylated p40 interacted with phosphorylated AKT resulting from the increased blood glucose in rat cardiac muscle

Insulin induced by increase blood glucose level has been shown to be involved in the activation of several cytoplasmic protein kinases such as Akt, starting the metabolic signaling pathways in muscles. To test this, we prepared the lysates from several muscles (cardiac muscle, soleus, and EDL) of wistar rats at 30 min after glucose intraperitoneal injection (GT1 and GT2) or no glucose injection (C1 and C2). The glucose levels of these rats are described in Fig. 1D. The lysates were analyzed by anti-phospho Akt antibody immunoblotting. Akt in soleus and EDL were phosphorylated in each case. In contrast, the phosphorylation level of Akt in cardiac muscle was upregulated at high blood glucose level, whereas the expression levels of Akt in cardiac muscles were similar (Fig. 1A). The bands just below the phospho-Akt bands did not cross-react with anti-Akt antibody. Therefore, these are non-specific bands which cross-react with phospho-Akt antibody. These results indicated that blood glucose level determined a good part of the phosphorylation level of Akt in cardiac muscle. It is highly likely that the downstream cascade of Akt in cardiac muscle was different from that in others, so we investigated what interacts with phosphorylated Akt in cardiac muscle. The lysates from cardiac muscles of C1, C2, GT1, GT2 were immunoprecipitated with anti-Akt antibody, and the precipitates were analyzed by anti-phospho Ser/Thr/Tyr antibody immunoblotting. In particular, the degree of phosphorylation of ~40 kDa protein (p40) interacted with Akt was markedly increased in GT1 cardiac muscle (Fig. 1B, upper panel, arrow). In contrast, the amounts of the band in C1, C2 and GT2 cardiac muscles were marginal. Despite the fact that it was a glucose-injected rat, GT2 cardiac muscle did not show a reaction similar to GT1. This is because this reaction may correlate with the blood glucose levels. Reprobing of the blot for Akt revealed that the amounts of Akt in each sample were very similar. Thus, we speculate that this protein is a novel target of Akt.

3.2. Identification of p40

p40 was partially purified and molecular mass analyses were performed as described in Section 2. According to this data, p40 represented GAPDH (Table 1). Therefore, we conducted experi-

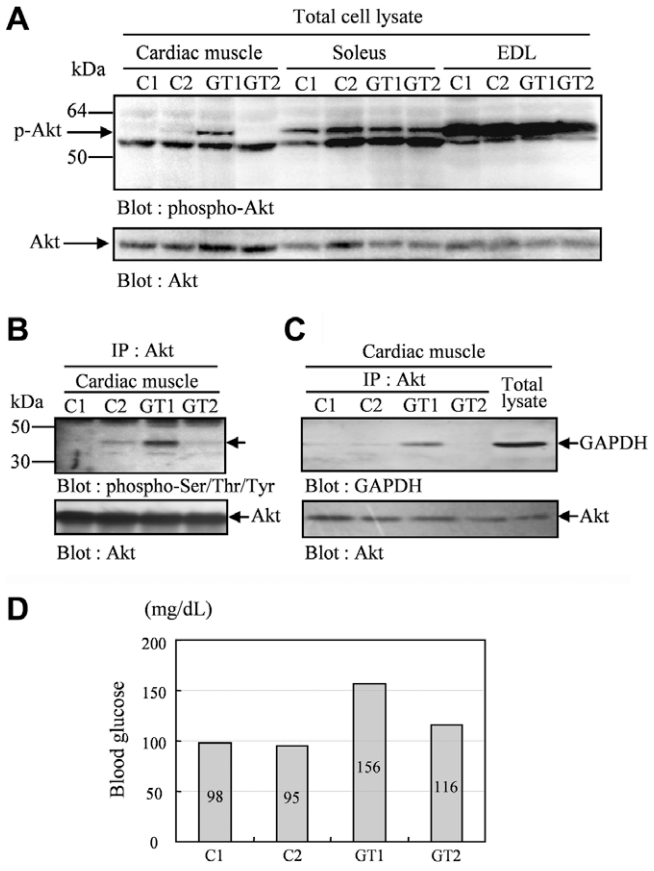

Fig. 1. Phosphorylated p40 interacts with Akt after glucose injection in cardiac muscle. Cardiac muscle, soleus, and EDL were removed from the rats at 30 min after glucose injection (GT1 and GT2) or no injection (C1 and C2). Representative results of separate experiments are shown in (A–D). (A) Lysates from muscles were prepared and analyzed by anti-phospho Akt and then anti-Akt blot. (B) Lysates from muscles were prepared and immunoprecipitated with anti-Akt antibody. Immunoprecipitates (IPs) were analyzed by anti-phospho-Ser/Thr/Tyr and then anti-Akt blot. (C) Lysates from cardiac muscle were prepared and immunoprecipitated with anti-Akt antibody. IPs were analyzed by anti-GAPDH and then anti-Akt blot. (D) Blood glucose concentrations were measured by blood glucose monitor.

Table 1
Molecular mass analysis of trypsin peptides.

Observed mass (Da)	Theoretical mass (Da)	Δ mass (Da)	Sequences	Positions
2244.12	2244.09	0.03	R VIISAPSADAPMFVMGVNHEK Y	117–137
738.35	738.35	0.00	K YDNSLK I	138–143
2610.42	2610.35	0.07	K VIHDNFGIVEGLMTTVHAITATQK T	161–184
1368.76	1368.74	0.02	K LTGMAFR V	199–213
810.40	810.41	0.00	K LTGMAFR V	226–232
1569.85	1569.82	0.03	R VPTPNVSVVDLTCR L	233–246
812.43	812.44	0.01	K QAAEGPLK G	262–269
1778.82	1778.79	0.03	K LISWYDNEYGYSNR V	308–321
1258.61	1258.59	0.02	R VVDLMAYMASK E	322–332

Match to: GAPDH.
Sequence coverage: 36%.

ments to confirm that p40 was GAPDH. The lysates from cardiac muscles of C1, C2, GT1, GT2 were immunoprecipitated with anti-Akt antibody, and the precipitates were investigated by anti-GAPDH antibody blot experiments. The result shown in Fig. 1C indicates that Akt interacts with GAPDH upon blood glucose level increase in cardiac muscle. This result suggests that Akt and GAPDH are linked not by glucose injection time but by blood glucose level.

3.3. The relationship between phosphorylation level of GAPDH and blood glucose level

To investigate the relationship between phosphorylation level of GAPDH and blood glucose level, the lysates from cardiac muscles of wistar rats at 30, 60, 120 min after glucose intraperitoneal injection ($GT3_{30min}$, $GT4_{60min}$, and $GT5_{120min}$) or no glucose injection (C3) were immunoprecipitated with anti-GAPDH antibody, and the precipitates were examined by anti-phospho Ser/Thr/Tyr antibody. The glucose levels of these rats are described in Fig. 2B. The result demonstrated that GAPDH phosphorylation was upregulated at high blood glucose level after glucose injection, then GAPDH phosphorylation returned to the original level followed by the decrease of blood glucose level within 120 min in cardiac muscle (Fig. 2A). The phosphorylation of GAPDH was marginal on the muscles (soleus and EDL) other than cardiac muscle regardless of the change of the blood glucose level (data not shown).

3.4. GAPDH interacts with AKT after insulin stimulation in H9c2 cell line

In order to investigate whether Akt interacts with GAPDH after insulin stimulation, we used a H9c2 cell line derived from embryonic rat heart that can serve as a surrogate for cardiac muscle in vitro. H9c2 cells resuspended in PBS were treated with, or without, insulin (final concentration: 100 nM). The lysates were analyzed by anti-phospho Akt antibody immunoblotting. Phosphorylation of Akt was induced by 15 min insulin treatment (Fig. 3A). The phosphorylation level was sustained for 30 min (data not shown). On the other hand, phosphorylation of Akt diminished

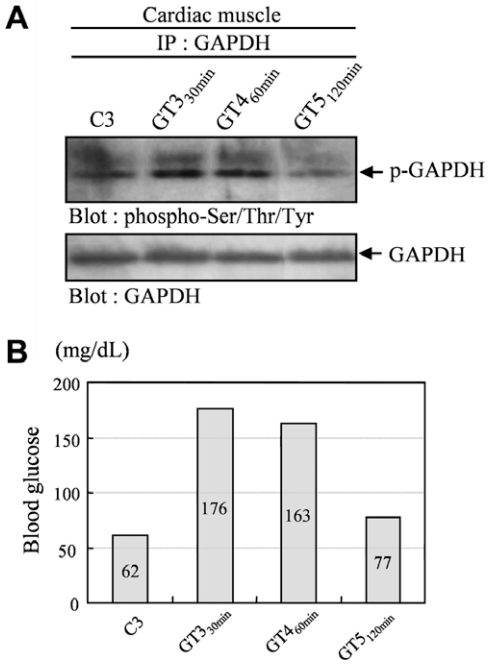

Fig. 2. Relationship between phosphorylation level of GAPDH and blood glucose level. Representative results of separate experiments are shown in (A) and (B). (A) Cardiac muscle was removed from each rat at 30 min ($GT3_{30min}$), 60 min ($GT4_{60min}$), 120 min ($GT5_{120min}$) after glucose injection and from control rats (no glucose injection) (C3). Lysates were prepared and immunoprecipitated with anti-GAPDH antibody. IPs were analyzed by anti-phospho-Ser/Thr/Tyr and then anti-GAPDH blot. (B) Blood glucose concentrations were measured by blood glucose monitor at every time point.

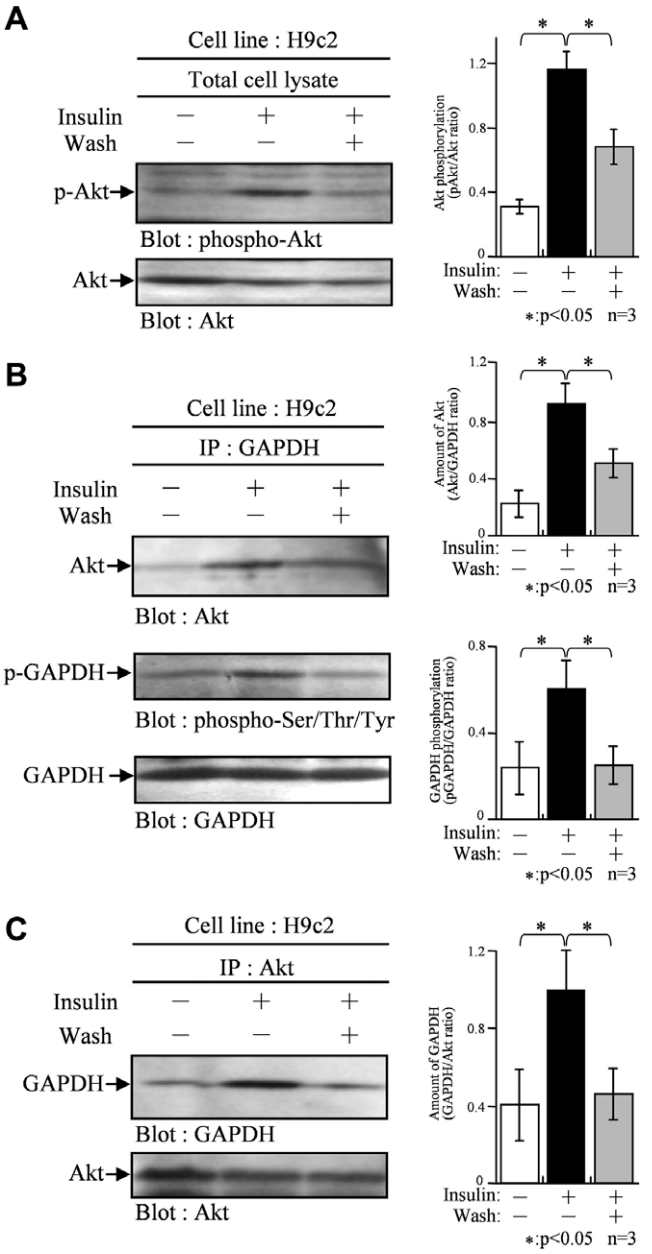

Fig. 3. GAPDH Interacts with AKT after insulin stimulation in H9c2 cell line. H9c2 cells were treated with or without insulin and then had insulin flushed out with PBS after 15 min insulin treatment. Blots representative of separate experiments are shown. Evaluation data of band densities of the immunoblots using Image J software are expressed as means ± S.E.M. for 3 independent experiments in all cases. (A) Lysates were prepared and analyzed by anti-phospho Akt and then anti-Akt blot. (B) Lysates were prepared and immunoprecipitated with anti-GAPDH antibody. IPs were analyzed by anti-AKT or anti-phospho-Ser/Thr/Tyr blot. The blot was then reprobed with anti-GAPDH antibody. (C) Lysates were prepared and immunoprecipitated with anti-Akt antibody. IPs were analyzed by anti-GAPDH and then anti-Akt blot.

in the cells which were flushed out of insulin with PBS after 15 min insulin treatment (Fig. 3A). These results indicate that insulin determines the phosphorylation status of Akt. Furthermore, phosphorylation level of GAPDH was upregulated with increase of Akt phosphorylation after 15 min insulin treatment, and then, phosphorylated GAPDH was reduced concomitantly with the decrease of phosphorylated Akt after washing away insulin (Fig. 3B). Moreover, more GAPDH molecules were interacted with Akt when both of them were hyper-phosphorylation status (Fig. 3B and C). The results suggest that phosphorylation status of GAPDH and interaction between Akt and GAPDH correlate closely with phosphorylation status of Akt after insulin stimulation in H9c2 cell line.

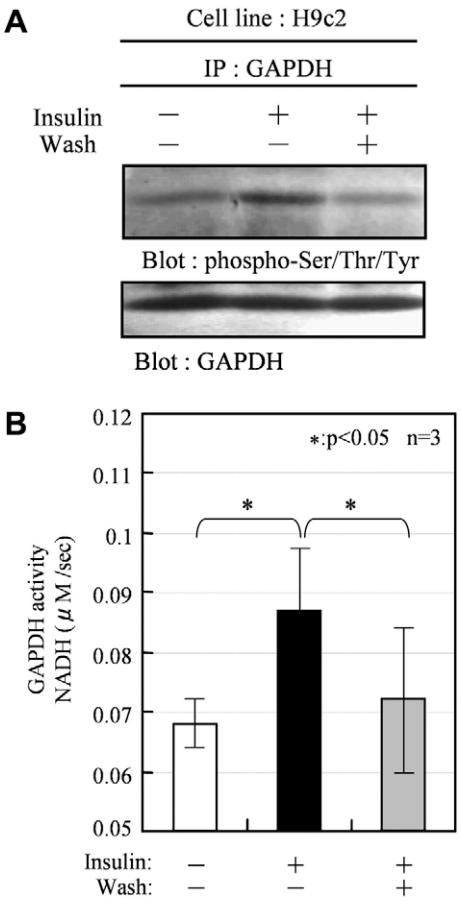

Fig. 4. The effects of insulin on GAPDH activity. H9c2 cells resuspended in PBS were treated with or without insulin and then had insulin flushed out with PBS after 15 min insulin treatment. Blots representative separate experiments are shown. (A) Lysates were prepared and immunoprecipitated with anti-GAPDH antibody. IPs were analyzed by anti-phospho-Ser/Thr/Tyr and then anti-GAPDH blot. (B) The reactions were conducted as already described in Section 2. The measurements were repeated 3 times for each of the 3 experiments. Results are shown as means ± S.E.M.

3.5. The relationship between phosphorylation status of GAPDH and activity of GAPDH

To investigate the relationship between phosphorylation status of GAPDH and activity of GAPDH, GAPDH activity was determined in whole lysates taken from H9c2 cell lines treated with, or without, insulin. GAPDH exhibited greater activity with the increased phosphorylation level of GAPDH after insulin stimulation, and then, GAPDH activity was reduced concomitantly with the decrease of phosphorylated GAPDH after washing away insulin in the cell line (Fig. 4A and B). Therefore, the results suggest that the phosphorylation of GAPDH has some kind of connection with GAPDH activity.

4. Discussion

Insulin participates in the regulation of glucose uptake, protein synthesis, and vascular tonicity. Among the signal transduction mechanisms involved in these insulin effects, Akt pathway is thought to play a crucial role. However, the full identification of

insulin-induced activation has not yet been actually demonstrated in cardiac tissue.

To investigate the downstream cascade of Akt in cardiac muscle, we used several rat muscles after glucose intraperitoneal injection. In cardiac muscle, phosphorylation of Akt had a direct correlation with blood glucose level compared with soleus and EDL after glucose injection. We speculate that Akt in soleus and EDL were phosphorylated regardless of glucose injection because Akt is situated at a critical juncture where it responds to inhibition of apoptosis and protein degradation in skeletal muscle. Phosphorylation of p40 was interacted with Akt in cardiac muscle after glucose injection. This protein turned out to be GAPDH. Furthermore, the interaction between GAPDH and Akt was induced by insulin stimulation in H9c2 cell line derived from embryonic rat heart. These results suggest that there is a novel Akt/GAPDH signal cascade not in skeletal muscle but in cardiac muscle. GAPDH is one of the abundant proteins in the cell. Therefore, we ruled out the possibility of non-specific detection of the Akt/GAPDH complex (Fig. 3). Akt phosphorylates a wide array of downstream effector proteins. Therefore, our data suggest that Akt activated by insulin may phosphorylate GAPDH as new substrate.

GAPDH is commonly known as an enzyme that catalyzes the sixth step of glycolysis (GAPDH catalyzes the NAD-mediated oxidative phosphorylation of glyceraldehyde 3-phosphate to 1,3-diphosphoglycerate) and thus serves to break down glucose for energy and carbon molecules. Recent reports demonstrate that GAPDH has been implicated in several non-metabolic processes. Hara, et al. demonstrated that GAPDH is S-nitrosylated by NO in response to cell stress, which causes it to bind to a ubiquitin ligase, Siah1, and initiates apoptosis [13]. Therefore, the modification status of GAPDH may play a pivotal role in its function. Another paper suggests that GAPDH is phosphorylated by PKC ι/λ and that it plays a role in microtubule dynamics in the early sectetory pathway in kidney cells [14]. However, the relationship between phosphorylation status of GAPDH and activity of GAPDH has remained unknown. Therefore, to investigate this relationship, activity of phosphorylated GAPDH induced by insulin was determined by analysis for the presence of NADH from NAD^+. We demonstrated that GAPDH has greater activity in response to the increase of phosphorylation level of GAPDH in the H9c2 cell line after insulin stimulation.

In conclusion, we suggest that insulin treatment triggers GAPDH phosphorylation and association with Akt, and that GAPDH phosphorylation in response to insulin stimulation may correlate with the enhancement of GAPDH enzymatic activity. These results demonstrate for the first time the interaction of Akt with GAPDH in cardiac muscle.

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