Evidence against high glucose as a mediator of ERK1/2 or p38 MAPK phosphorylation in rat skeletal muscle

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Kawano, Yuichi, Jeffrey W. Ryder, Jorge Rincon, Juleen R. Zierath, Anna Krook, and Harriet Wallberg-Henriksson. Evidence against high glucose as a mediator of ERK1/2 or p38 MAPK phosphorylation in rat skeletal muscle. Am J Physiol Endocrinol Metab 281: E1255–E1259, 2001.—Hyperglycemia leads to multiple changes in insulin signaling in skeletal muscle from people with type 2 diabetes. We hypothesized that mitogen-activated protein kinase (MAPK) signaling cascades may be directly activated by an acute exposure to high extracellular glucose concentrations. We determined whether an elevation in the extracellular glucose concentration would induce signal transduction in skeletal muscle via MAPK cascades. Epitrochlearis muscles were incubated in the presence of 5 or 25 mM glucose. Exposure of muscle to either hyperosmosis (600 mM mannitol) or insulin (6 nM) led to a marked increase in extracellular signal-regulated protein kinase (ERK1/2) phosphorylation. Hyperosmosis elicited a 5.2-fold increase in p38 phosphorylation (P < 0.05), whereas insulin was without effect. ERK1/2 phosphorylation was not increased by high glucose exposure. After a 20-min exposure to 25 mM glucose, a tendency toward repressed (23%) p38 phosphorylation was observed (P = 0.06). No effect of high glucose was noted on signal transduction to signal transducer and activator of transcription 3 and Akt. In conclusion, short-term exposure of skeletal muscle to high levels of glucose does not appear to alter ERK1/2 or p38 MAPK phosphorylation.

extracellular signal-regulated protein kinase 1 and 2; hyperglycemia; hyperosmosis; insulin; protein kinase C; Akt/protein kinase B; skeletal muscle; signal transducer and activator of transcription 3; cellular signaling; insulin receptor substrate 1; c-jun NH2-terminal protein kinase

A GROWING BODY OF EVIDENCE suggests that hyperglycemia plays a significant role in the development of peripheral insulin resistance (31, 33–35, 43). We (44) have previously shown that restoration of glycemia normalizes the reduced capacity for insulin-stimulated glucose transport in skeletal muscle from people with type 2 diabetes mellitus. Impaired insulin signal transduction has been noted in skeletal muscle from obese insulin-resistant (10) or moderately obese people with type 2 diabetes mellitus (3, 19, 20). This aberrant signal transduction may be a consequence of hyperglycemia characteristic of the diabetic phenotype. In skeletal muscle, key components of the insulin signal transduction cascade are sensitive to elevated levels of glycemia (22, 39). Thus hyperglycemia may directly contribute to the development of insulin resistance in people with type 2 diabetes through altered insulin signal transduction in peripheral tissues.

The mechanism(s) by which hyperglycemia activates intracellular signaling cascades remains largely unknown. Several lines of evidence implicate protein kinase C (PKC) as a mediator of hyperglycemia-induced signal transduction (14, 16, 29, 31, 40, 41). Acute hyperglycemia has also been shown to directly increase plasma membrane GLUT-4 content (9), providing evidence for a glucose-dependent autoregulation of glucose transport in skeletal muscle. We have shown that the glucose-induced increase in transport activity is blocked by dantrolene, an inhibitor of Ca2+ release from the sarcoplasmic reticulum (30), and may involve the activation of PKCδ2 (16). In contrast, inhibition of phosphatidylinositol (PI) 3-kinase by wortmannin is without effect on the glucose-induced increase in glucose transport (30). These studies provided evidence that hyperglycemia is a direct activator of intracellular signal transduction.

The mitogen-activated protein kinase (MAPK) family constitutes a major ubiquitous intracellular signaling system involved in the regulation of cell growth, differentiation, and survival (37). The extracellular signal-regulated protein kinases 1 and 2 (ERK1/2) are activated by hormones and growth factors via a Ras-dependent signal transduction pathway and have been implicated in control of cellular proliferation and differentiation by stimulation of transcription factors that induce the expression of growth-responsive genes (2, 21). Hyperglycemia may lead to impaired insulin signal transduction via activation of the MAPK signaling cascade. MAPK has recently been reported to phosphorylate the insulin receptor substrate 1 (IRS-1), a primary target in the insulin signaling cascade, leading to impaired activity of downstream components of the insulin signal transduction cascade (6).
Two additional parallel signal transduction pathways, c-jun NH₂-terminal protein kinase (JNK) and p38 MAPK, have been identified (7, 12, 23, 24). These pathways are activated in response to environmental stress factors, including ultraviolet light (7, 12), osmotic stress (28, 38), heat shock, and proinflammatory cytokines (4, 5, 8, 11, 13, 15, 18, 27, 32, 36, 42). Hyperglycemia leads to increased p38 phosphorylation in rat aortic smooth muscle cells (15) and increased ERK phosphorylation in rat glomeruli and mesangial cells (13). Thus we hypothesized that an acute exposure of skeletal muscle to high glucose may activate signal transduction via MAPK signaling cascades.

MATERIALS AND METHODS

Materials. Chemicals were purchased from Sigma Chemical (St. Louis, MO). The insulin (Actrapid) was a product of Novo-Nordisk (Copenhagen, Denmark). Materials for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) were from Bio-Rad (Richmond, CA). All antibodies were from New England Biolabs (Beverley, MA).

Animals. Male Wistar rats (95–115 g) were purchased from B&K Universal (Sollentuna, Sweden) and were housed in the animal facility at the Karolinska Hospital for 1 wk before experiments. All rats were maintained in a climate-controlled environment with a 12:12-h alternating light-dark cycle. Rats received standard rodent chow and water ad libitum.

Muscle dissection and incubation. Overnight-fasted rats were anesthetized with an intraperitoneal injection of pentobarbital sodium (5 mg/100 g body wt). Thereafter, epitrochlearis muscles were carefully dissected out. Muscles were initially incubated for 15 min in sealed glass flasks containing Krebs-Henseleit bicarbonate (KHB) buffer supplemented with 0.1% BSA (RIA grade), 5 mM HEPES, and 40 mM mannitol. Thereafter, muscles were incubated for 2–40 min in KHB containing 5 mM glucose (basal), 25 mM glucose (hyperglycemia), 600 mM mannitol (hyperosmosis), or 6 nM insulin. The concentration of insulin used in the present study is known to elicit a maximal effect on glucose transport in rodent skeletal muscle (8). Each vial was continuously gassed with 95% O₂-5% CO₂ and maintained in a shaking water incubator (30°C). Mannitol was included in all media to adjust for changes in osmolarity between basal and glucose conditions (total of 40 mM). Muscles were snap frozen in liquid nitrogen and stored at −80°C until analysis.

Preparation of total muscle homogenates. Muscles were homogenized (4°C) in buffer [50 mM HEPES pH 7.6, 150 mM NaCl, 1% Triton X-100, 1 mM Na₂VO₄, 10 mM NaF, 30 mM Na₃P₂O₇, 10% (vol/vol) glycerol, 1 mM benzamidine, 1 mM dithiothreitol, 10 μg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride], and lysates were cleared by centrifugation at 15,000 g for 15 min (4°C).

Western blot analysis. Protein concentration was determined using a kit from Bio-Rad. Aliquots (30 μg) were solubilized in Laemmli buffer, separated by SDS-PAGE (7.5% resolving gel), and transferred to polyvinylidene difluoride membranes as described (39). Membranes were blocked overnight at 4°C in TBST (5% nonfat milk in 10 mM Tris-HCl, 100 mM NaCl, and 0.1% Tween-20, pH 7.6). Membranes were exposed to the primary antibodies, followed by washing (3 × 15 min with TBST). To determine ERK1/2 phosphorylation, membranes were immunoblotted with a phosphospecific p42/44 MAPK antibody that recognizes p42 MAPK phosphorylated at Thr180 and Tyr182. Phosphorylation of protein kinase B (Akt) was assessed using a phospho-Akt antibody that recognizes Akt kinase when phosphorylated at Ser473. Phosphorylation of signal transducer and activator of transcription 3 (STAT3) was assessed using a phospho-STAT3 antibody that recognizes STAT3 when phosphorylated at Tyr705. The phosphospecific antibodies were purchased from New England Biolabs. Membranes were incubated with horseradish peroxidase-conjugated anti-mouse or anti-rabbit IgG antibody, followed by washing with TBST (3 × 15 min). Proteins were visualized by enhanced chemiluminescence (Amersham, Arlington Heights, IL) and quantified by densitometry.

Statistics. For all experiments, one muscle from each animal was incubated under basal conditions, and the contralateral muscle was incubated in the presence of hyperglycemia, hyperosmosis, or insulin. Results are presented as increase over basal. All data are expressed as mean amounts of difference over basal ± SE. Statistical analysis was performed using the unpaired Student’s t-test.

RESULTS

Effects of elevated glucose levels on ERK1/2 and p38 MAPK. Isolated epitrochlearis muscle was incubated for 2, 7, 12, 20, or 40 min in KHB media containing 5 or 25 mM glucose. Animal-to-animal variability in basal MAPK phosphorylation was observed (range 1.6–19.8 arbitrary units; mean ± SE basal phosphorylation 8.02 ± 0.29 arbitrary units; n = 19). Thus paired muscles were used in all experiments, with one epitrochlearis muscle from each animal incubated in media containing 5 mM glucose and the contralateral muscle incubated with 25 mM glucose. We have previously shown that an acute 35-min exposure of skeletal muscle to high levels of glucose leads to activation of glucose transport and the translocation of PKCβ2 from the cytosol to the total membrane fraction (16). However, in the present study, exposure to high glucose did not lead to a significant phosphorylation of ERK1/2 at any of the time points tested (data not shown). We further assessed whether glucose altered p38 phosphorylation. A tendency toward reduced (23%) p38 phosphorylation was observed after 20 min of exposure to high glucose, although this did not reach statistical significance (P = 0.06, Fig. 1).

Effect of hyperosmolarity and insulin on ERK1/2 and p38 MAPK. Insulin and hyperosmosis increase phosphorylation and activation of MAPK in several tissue culture systems (17). Epitrochlearis muscle was incubated with 600 mM mannitol (hyperosmosis) or insulin (6 nM) for 20 min, and MAPK phosphorylation was determined. A significant increase in ERK1/2 phosphorylation was observed in muscle exposed to either hyperosmosis or insulin (Fig. 2A). Hyperosmosis induced a profound 5.2-fold increase in p38 phosphorylation (P < 0.05; Fig. 2B) in skeletal muscle. In contrast, insulin did not alter p38 phosphorylation (data not shown).
Acute exposure of rat epitrochlearis muscle to high levels of glucose did not alter phosphorylation of either Akt kinase or STAT3 (Fig. 3).

**Effect of elevated glucose levels on Akt kinase or STAT3.** To assess whether glucose has a direct effect on other cellular signaling cascades, we incubated rat epitrochlearis muscle in the presence of 5 or 25 mM glucose and determined phosphorylation of Akt kinase or STAT3. Epitrochlearis muscle was exposed to 25 mM glucose for 20 min. Phosphorylation of Akt was assessed using a phospho-Akt antibody that recognizes Akt when phosphorylated at Ser^473. Phosphorylation of STAT3 was assessed using a phospho-STAT3 antibody that recognizes STAT3 when phosphorylated at Tyr^705. Acute exposure of rat epitrochlearis muscle to high levels of glucose did not alter phosphorylation of either Akt kinase or STAT3 (Fig. 3).

**DISCUSSION**

We have previously demonstrated (44) that, after a short-term (2-h) incubation of skeletal muscle from insulin-resistant type 2 diabetic patients in normoglycemic media, insulin-stimulated glucose transport is restored to nondiabetic levels. Thus hyperglycemia has a profound and deleterious direct effect on insulin action in skeletal muscle. We hypothesized that members of the MAPK family were targets for hyperglycemia-induced signal transduction, since glucose-induced MAPK phosphorylation may lead to reduced insulin signal transduction (6). Here, we show that acute exposure of skeletal muscle to high levels of extracellular glucose does not increase phosphorylation of ERK1/2 or p38 MAPK. In contrast, we show that acute exposure to hyperosmosis increased ERK1/2 and p38 MAPK phosphorylation and acute insulin exposure increased ERK1/2 phosphorylation in our isolated epitrochlearis muscle preparation.

Several reports provide evidence that high glucose exposure leads to the activation of various MAPK cascades in pancreatic cell lines (8, 17). In the insulinoma cell line INS-1, activity/phosphorylation of ERK1/2 and p38 increase in response to glucose exposure (8, 17). Furthermore, in MIN6 pancreatic β-cells, glucose-induced activation of ERK1/2 has been observed (1). Activation of ERK1/2 does not appear to be required for glucose-mediated insulin secretion, as the MAPK inhibitor PD-098059 did not block this effect (1, 17). However, because glucose stimulation has been reported to be associated with ERK translocation to the nucleus (1), a glucose-mediated, calcium-dependent transcriptional response of cells may regulate expression of the insulin gene. High glucose also activates p38 in MIN6 cells, leading to the activation of insulin upstream factor 1, a transcription factor that binds to sequences present in the human insulin promoter (25).

In rat aortic smooth muscle cells, long-term (72-h) exposure to elevated glucose levels increases p38 MAPK activity (15). Furthermore, long-term (5-day) exposure of mesangial cells to 27.8 mM glucose leads to a significant increase in MAPK activity (13). In contrast, inhibition of MAPK phosphorylation has been observed in mesangial cells after a hyperglycemic chal-
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