Communication

Signaling Pathway Involved in the Activation of Heart 6-Phosphofructo-2-kinase by Insulin

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Incubation of isolated rat cardiomyocytes with insulin increased 2-deoxyglucose uptake, glycogen synthesis, and fructose 2,6-bisphosphate content. Half-maximal effects were obtained with 1–2 nm insulin. The insulin-induced increase in fructose 2,6-bisphosphate content was preceded by a 2–3-fold activation of 6-phosphofructo-2-kinase, which was independent of glucose transport. Insulin activated phosphatidylinositol 3-kinase and p70 ribosomal S6 kinase (p70 S6 kinase), but had no significant effect on mitogen-activated protein kinase, although phosphorl 12-myristate 13-acetate activated the latter. The effect of insulin on fructose 2,6-bisphosphate, 6-phosphofructo-2-kinase, and phosphatidylinositol 3-kinase was blocked by wortmannin. However, rapamycin, which inhibited p70 S6 kinase activation, and PD 98059, an inhibitor of the mitogen-activated protein kinase pathway, had no effect on the insulin-induced activation of 6-phosphofructo-2-kinase. Heart 6-phosphofructo-2-kinase can therefore be regarded as a glycolytic target of insulin. Its activation by insulin might be mediated by phosphatidylinositol 3-kinase.

Heart glycolysis is regulated by substrate supply, energy demand, and hormones such as adrenaline and insulin (1, 2). Fructose 2,6-bisphosphate (Fru-2,6-P₂) is a positive effector of 6-phosphofructo-1-kinase and participates in the regulation of glycolysis in this tissue (3–6). Furthermore, intravenous injection of insulin to rats more than doubled heart Fru-2,6-P₂ content, which could not solely be explained by the rise in hexose 6-phosphate concentration (7). Insulin doubled the Vₘₐₓ of 6-phosphofructo-2-kinase (PFK-2), and the effect persisted following partial purification of the enzyme, suggesting that covalent modification might be involved in its activation (7). An insulin-induced increase in Fru-2,6-P₂ content has been confirmed in perfused hearts (4, 6), see however Ref. 8.

Diverse insulin signaling cascades have been proposed to mediate the metabolic effects of insulin (for reviews, see Refs. 9 and 10). One of these involves the activation of mitogen-activated protein kinase (MAPK, e.g. Erk-1 and -2 or extracellular signal-regulated kinase), via the activation of Ras, Raf, and MAPK kinase (MAPKK). Recently, a novel inhibitor of this pathway, PD 98059, has been reported to block the activation of MAPKK (11) and, hence, of MAPK and one of its downstream targets, p90 ribosomal S6 kinase. The second signaling pathway is triggered by the activation of phosphatidylinositol 3-kinase (PI 3-kinase) and leads to the activation of the p70 S6 kinase (12). Wortmannin inhibits PI 3-kinase (13, 14), whereas rapamycin blocks the activation of p70 S6 kinase (15, 16).

The aims of the present work were to validate the isolated cardiomyocyte preparation as a model to study insulin action on glycolysis and to investigate the insulin-signaling pathway leading to PFK-2 activation.

EXPERIMENTAL PROCEDURES

Materials

Radiochemicals (Amersham); wortmannin, phloretin, 2-deoxyglucose, myelin basic protein (MBP), and α-l-phosphatidylinositol (PI) (Sigma); rapamycin (Biomol); fatty acid-free fraction V bovine serum albumin and collagenase A (Boehringer Mannheim); anti-MAPK antibodies (anti-Erk-1 and anti-Erk-2) and anti-p70 S6 kinase antibodies (Santa Cruz); and anti-PI 3-kinase antibodies (U.B.I.) were obtained as indicated. PD 98059 was a generous gift from Prof. P. Cohen (University of Dundee, Scotland). Stock solutions of 10 mM wortmannin, 10 mM rapamycin, and 50 mM PD 98059 were stored at −20 °C in dimethyl sulfoxide (Me₂SO) and diluted in 0.9% NaCl before use. The cAMP-dependent protein kinase inhibitor peptide (TYADFIASGRTRGGRRNAHD) (17) and the small p70 S6 kinase peptide (RLRQLSLRA) (18) were synthesized by Dr. J. Lucchetti (Ludwig Institute, Brussels). All other biochemicals were from Sigma or Boehringer.

Preparation and Incubation of Isolated Cardiomyocytes

Isolated cardiomyocytes were prepared by the method of Fischer et al. (19) originally described for Sprague-Dawley rats and which we have also applied to Wistar rats. The perfusion medium was a Krebs-Henseleit medium without CaCl₂ and supplemented with 5 mM glucose, 2 mM pyruvate, and 10 mM Hepes (pH 7.4) in equilibrium with O₂/CO₂ (95%, 5%). Two hearts were perfused in parallel, and the cardiomyocytes from both hearts were resuspended in the same buffer to obtain a single preparation. The cardiomyocytes (about 20 to 30 mg wet weight of cells/ml) were incubated at 37 °C in 20-ml vials with constant agitation in the presence of the indicated additions and in a medium containing 134 mM NaCl, 4.7 mM KCl, 1.25 mM MgSO₄, 1.2 mM KH₂PO₄, 12 mM Hepes at pH 7.4, 1 mM CaCl₂, 2% bovine serum albumin, and, except where otherwise stated, 5 mM glucose. When cardiomyocytes were incubated in the presence of wortmannin, rapamycin, or PD 98059, untreated cells received the same amount of Me₂SO. The wet weight of each cell preparation was measured by weighing the cell pellet after centrifugation (10,000 × g for 30 s).

PMA, phorbol 12-myristate 13-acetate.
The yield of cardiomyocytes was about 8 × 10^6 cells from a single heart. Sprague-Dawley rats and about 45% less from a Wistar rat. By trypan blue exclusion, cell viability was estimated to be more than 95%, among which about 80% were nonbeating, rod-shaped cells. The overall effects of insulin on 2-deoxyglucose transport, glycogen synthesis, and Fru-2,6-P₂ content were qualitatively the same in cardiomyocytes from both rat strains, although the magnitude of the insulin effect was 30–50% greater in Sprague-Dawley than in Wistar cardiomyocytes. Most of the studies reported here were performed with Wistar rats because this strain has been used for years in our laboratory.

Perfusion

Hearts from Wistar rats were perfused with Krebs-Henseleit buffer in the presence of 5 mM glucose and 1.25 mM CaCl₂ for 10 min (5, 20) without or with 0.25 μM rapamycin, followed by a 30-min perfusion with or without 0.1 μM insulin. At the end of the perfusion, the hearts were freeze-clamped between aluminium blocks precooled in liquid nitrogen, for measurement of Fru-2,6-P₂ content and enzyme assays.

Metabolic Fluxes and Fru-2,6-P₂ Content

Glycogen synthesis was measured by the incorporation of 5 mM [1-¹⁴C]glucose (40 μCi/mmol) into glycogen in cardiomyocytes incubated for 30 min (21). The amount of 2-[2-³H]deoxyglucose (1 μM; 100 mCi/mmol) was taken up by the cells in a 20-min incubation without glucose was measured as described (19). Fru-2,6-P₂ content was measured (22) in cells incubated for the indicated periods of time in the presence of 5 mM glucose, except where otherwise stated. Glycolytic flux was measured by the detritiation of [³H]glucose (6), which gives an estimation of the flux through 6-phosphofructo-1-kinase.

Enzyme Assays

PFK-2—For the measurement of PFK-2 activity, cell pellets (about 30 mg of cells) were resuspended in 1 ml of cold 20% Heps buffer, pH 7.5, 30 mM KCl, 20 mM KF, 5 mM EDTA, 1 mM dithiothreitol, 0.1 mM phenylmethanesulfonyl fluoride, 0.1 mM fructose 6-phosphate, 0.3 mM glucose 6-phosphate and frozen in liquid N₂. The cells were thawed and homogenized at 4 °C with an Ultra-Turrax (3 × 20 s). After centrifugation (10,000 × g for 5 min), 5–20% polyethylene glycol (PEG) 6000 fractions were prepared from four pooled cell preparations. This PEG fraction was assayed for lactate dehydrogenase (LDH) (23) and PFK-2. PFK-2 was assayed at pH 7.1 in the presence of 2 mM fructose 6-phosphate and 5 mM MgATP (7). For PFK-2 measurement in perfused hearts, frozen samples were homogenized in 5 volumes of homogenization buffer (see above) for PEG fractionation. MAP Kinase—MAP kinase was measured by the phosphorylation of MBP in immunoprecipitates from cardiomyocyte extracts. The homogenization procedure, immunoprecipitation (10 μl of antibody for 5–10 mg of cells) of MAPK, and the assay conditions were as described (24). The assay was performed on immunoprecipitates corresponding to 5–10 mg of cells. MBP was separated by SDS-PAGE in gels containing 15% (w/v) acrylamide. Gels were dried and autoradiographed. Radioactivity incorporated into MBP was measured by phosphorimaging (Molecular Dynamics). The results are expressed as percentage of control values.

p70 S6 Kinase—p70 S6 kinase was assayed in immunoprecipitates from perfused hearts by phosphorylation of the small ribosomal S6 peptide (RRRLSSLRA) (24, 25). Each heart was homogenized (4 × 20 s, Ultra-Turrax) in 5 volumes (w/v) of buffer containing 20 mM sodium β-glycerophosphate, pH 7.2, 20 mM NaF, 2 mM EDTA, 1 mM NaVO₄, 1 mM benzamidine-Cl, 4 μg/ml leupeptin, 0.2 mM phenylmethanesulfonyl fluoride, 0.3% (v/v) β-mercaptoethanol. After centrifugation (5000 × g for 20 min), a 5% (w/v) PEG 6000 fraction was prepared and resuspended in 1 ml of homogenization buffer for each heart. p70 S6 kinase present in 0.5 ml of this fraction was immunoprecipitated with 10 μl of anti-p70 S6 kinase antibodies bound to Protein A-Sepharose beads and resuspended in homogenization buffer. p70 S6 kinase was assayed (24) with ³²P incorporation into small S6 peptide measured as described (24, 25).

PI 3-Kinase—PI 3-kinase activity was measured in immunoprecipitates from homogenates of cardiomyocyte pellets (20 mg of cells). Preparation of homogenates, immunoprecipitation (0.5 μl of antibody for 10–15 mg of cells), and assay conditions were as described (24). The assay was performed on immunoprecipitates corresponding to 10–15 mg of cells. [³²P]Phosphatidylinositol 3-phosphate was separated by TLC and radioactivity was measured by phosphorimaging.

Expression of Results

One unit of enzyme activity corresponds to the formation of 1 pmol of product/min under the assay conditions. The activity of PFK-2 in mi-
after continued during 30 min of incubation. This rise in Fru-2,6-P₂ correlated with an increase in flux through 6-phosphofructo-1-kinase as measured by detritiation of [3-¹³C]glucose (Fig. 1B). The insulin-induced activation of PFK-2 was independent of glucose transport, since a similar activation was observed when the cells were incubated with fructose or without a carbohydrate substrate (results not shown). The concentration of insulin giving half-maximal effect was 1.7 ± 0.2 nM for 2-deoxyglucose uptake, 1.0 ± 0.1 nM for the increase in glycogen synthesis, and 1.4 ± 0.4 nM for the increase in Fru-2,6-P₂ content (n = 3).

Effect of Wortmannin, Rapamycin, and PD 98059—The mechanisms of insulin action in cardiomyocytes were investigated. The effects of wortmannin on the insulin-induced stimulation of 2-deoxyglucose transport, glycogen synthesis, and Fru-2,6-P₂ content were studied in cells preincubated for 15 min with various concentrations of wortmannin before the addition of 0.1 μM insulin. Wortmannin inhibited the insulin-induced stimulation of 2-deoxyglucose transport, glycogen synthesis, and Fru-2,6-P₂ content, and the effect was maximal between 300 and 1000 nM wortmannin (Fig. 2). The concentration of wortmannin giving half-maximal inhibition was about 60–80 nM for the three parameters. The effect of insulin on PFK-2 activity was blocked by wortmannin, suggesting that PI 3-kinase activation is involved in the insulin-induced activation of PFK-2 (Table I).

By contrast, pretreatment of the cells with rapamycin did not affect the insulin-induced increase in glycogen synthesis and Fru-2,6-P₂ content (not shown). Likewise, the insulin-induced activation of PFK-2 was unaffected by rapamycin (Table I), suggesting that p70 S6 kinase activation is not required for this insulin effect. Moreover, preincubation with PD 98059 to prevent MAPK and MAPKAPK-1 activation or with the combination of rapamycin and PD 98059, to block both the p70 S6 kinase and MAPK branches of the insulin cascade, had no effect on PFK-2 activation (Table I).

To validate the results obtained with these inhibitors, we measured their effects on PI 3-kinase, Erk-2, and p70 S6 kinase activity. Insulin induced a 2-fold transient activation of PI 3-kinase, which was maximal at 1 min and disappeared at 5 min. This activation was completely inhibited by 0.3 μM wortmannin (Table I). Similarly, to ensure that rapamycin was active, we measured its effect on p70 S6 kinase activity. Since p70 S6 kinase activity was too low to be measured in cardiomyocytes, we studied its activation in perfused rat hearts. p70 S6 kinase activity was approximately tripled by insulin treatment (Fig. 3). In perfusions with 0.25 μM rapamycin, the insulin-induced activation of p70 S6 kinase was completely abolished. However, in these hearts, the insulin-induced activation of PFK-2 was unaffected by rapamycin, confirming that p70 S6 kinase is not required for the insulin-induced activation of PFK-2 (Fig. 3). A slight increase in PFK-2 activity by rapamycin treatment was observed in these experiments (Fig. 3), although no difference was found in isolated cardiomyocytes (Table I).

There was no significant effect of insulin on Erk-2 activity in cardiomyocytes incubated for 2 min (Table I) or 5 or 10 min (not shown) with insulin, confirming the lack of effect of rapamycin and PD 98059 on the insulin-induced activation of PFK-2 (Table I). A 3-fold increase in Erk-2 activity was observed in cardiomyocytes incubated with phorbol ester (results not shown). This is in agreement with previous findings (26) and shows that the MAPK cascade could be activated in our cell preparations. However, in cardiomyocytes incubated with phorbol ester, no change in Fru-2,6-P₂ content or PFK-2 activity was observed (results not shown), supporting in vitro studies showing that phosphorylation of purified heart PFK-2 by PKC had no effect on its activity (27).

**DISCUSSION**

The isolated cardiomyocyte model has enabled us to confirm not only the insulin-induced stimulation of glucose transport and glycogen synthesis (28, 29), but also the activation of PFK-2 and the resulting increase in Fru-2,6-P₂ content (4–7). Therefore, PFK-2 is a target of insulin action in the control of heart glycolysis. The activation of PFK-2 by insulin in isolated cardiomyocytes was not a consequence of the hormone-induced stimulation of glucose transport.

The effects of insulin to stimulate glucose uptake and glycogen synthesis were abolished by wortmannin, suggesting that PI 3-kinase activation mediates these processes (10, 11). Similarly, the insulin-induced increases in Fru-2,6-P₂ and PFK-2...
activity were abolished by wortmannin, implying that PI 3-kinase is involved. However, because the specificity of wortmannin for PI 3-kinase is subject to several observations (30), the evidence is only indirect. The activation of PFK-2 by insulin was unaffected by rapamycin and PD 98059, suggesting that activation of p70 S6 kinase, MAPK, or MAPKAPK-1 were not required for the insulin-induced activation of PFK-2.

The mechanism of activation of PFK-2 by insulin is probably the result of covalent modification. In vitro studies demonstrated that heart PFK-2 was phosphorylated and activated by the cyclic AMP-dependent protein kinase (27, 28). This activation resulted from a decrease in $K_m$ for fructose 6-phosphate (6, 27, 28). The involvement of this protein kinase in PFK-2 activation by insulin can be ruled out, because cyclic AMP is not required for the insulin-induced activation of PFK-2.

A likely candidate for mediating insulin action on PFK-2 is the novel Ser/Thr kinase, called protein kinase B or Akt/RAC (29, 31–34). This protein kinase is the cellular homologue of the viral oncogene v-akt (35, 36) and is activated through PI 3-kinase after insulin stimulation. Protein kinase B activation by insulin is indeed sensitive to wortmannin, but not to rapamycin. According to a recent study in cultured rat skeletal muscle L6 cells (32), Akt/RAC, but not p70 S6 kinase or MAPKAPK-1, is responsible for the insulin-induced inactivation of glycogen synthase kinase-3 and thus glycogen synthase activation. The possible phosphorylation and activation of heart PFK-2 by Akt/RAC is currently being studied in our laboratory.

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Fig. 3. Effects of rapamycin on p70 S6 kinase and PFK-2 activity in hearts perfused with or without insulin. Hearts were perfused with 5 mM glucose for 10 min with or without rapamycin (0.25 mM) and 30 min with or without insulin (0.1 mM). Each value represents the means ± S.E. of four separate experiments. * indicates a significant (p < 0.05) difference versus the corresponding control.
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