The hexokinases, by converting glucose to glucose 6-phosphate, help maintain the glucose concentration gradient that results in the movement of glucose into cells through the facilitative glucose transporters. Hexokinase II (HKII) is the major hexokinase isoform in skeletal muscle, heart, and adipose tissue. Insulin induces HKII gene transcription in L6 myotubes, and this, in turn, increases HKII mRNA and the rates of HKII protein synthesis and glucose phosphorylation in these cells. Inhibitors of distinct insulin signaling pathways were used to dissect the molecular mechanism by which HKII gene expression is induced by insulin in L6 myotubes. Treatment with wortmannin, an inhibitor of phosphatidylinositol 3-kinase (PI 3-kinase), or with rapamycin, an inhibitor of the pathway from the insulin receptor substrate-1 (IRS-1) to p70/p85 ribosomal S6 protein kinase (p70\(s6k\)), prevented the induction of HKII mRNA by insulin. In contrast, treatment with PD98059, an inhibitor of mitogen-activated protein kinase (MEK)-dependent protein kinase activation, had no effect on insulin-induced HKII mRNA. In addition, rapamycin blocked the insulin-induced expression of an HKII promoter-chloramphenicol acetyltransferase fusion gene transiently transfected into L6 myotubes, whereas PD98059 had no such effect. These results suggest that a phosphatidylinositol 3-kinase/p70\(s6k\)-dependent pathway is required for regulation of HKII gene transcription by insulin and that the Ras-mitogen-activated protein kinase-dependent pathway is probably not involved.

The four mammalian hexokinases (ATP:D-hexose-6-phosphotransferase, EC 2.7.1.1; designated HKI through IV) are a family of closely related enzymes that convert glucose to glucose 6-phosphate (for review, see Ref. 1). This enzymatic step initiates glucose metabolism and ensures the glucose concentration gradient that results in the movement of glucose into the cell through the facilitative glucose transporters (designated GLUT1 through 5) (2). Although HKI and HKII are both expressed in skeletal muscle and adipose tissue, HKII is the predominant isoform in these tissues (3). HKII catalytic activity is increased by insulin, whereas that of HKI is unaffected (3, 4). L6 myotubes have been used to analyze this action of insulin in these cells selectively increases HKII mRNA and protein synthesis, and this is associated with an increased rate of glucose utilization (4, 5). Hyperinsulinemic clamp studies have also demonstrated that HKII mRNA is increased by insulin in human and rat skeletal muscle in vivo (6, 7).

The binding of insulin to a specific cell-surface receptor results in the activation of an intrinsic receptor tyrosine kinase (for review, see Ref. 8). The activated receptor phosphorylates insulin receptor substrate-1 (IRS-1) and this phosphoprotein, through interaction with specific Src homology 2 (SH2) domain-containing proteins, initiates divergent signaling cascades (for review, see Ref. 9). For example, the enzyme phosphatidylinositol 3-kinase (PI 3-kinase; EC 2.7.1.67) is activated when it binds to IRS-1 (9). This leads to the accumulation of phosphatidylinositols phosphorylated at the 3-\(\alpha\) position of the inositol ring (10), and these molecules are presumed to act as second messengers (11). The antibiotic wortmannin, a potent inhibitor of PI 3-kinase (IC\(_{50}\)< 10 nM, in vitro), has been employed to examine the role of PI 3-kinase in a variety of insulin signaling pathways (see Refs. 12–21 and references therein). Wortmannin also blocks the activation of p70/p85 ribosomal S6 protein kinase (p70\(s6k\)) by insulin or insulin-like growth factor-1 (IGF-1), so it is presumed that p70\(s6k\) requires PI 3-kinase for its activation by these agents (13, 22, 23). Rapamycin, an immunosuppressive macrolide (for review, see Ref. 24), also blocks this action of insulin without affecting PI 3-kinase activity. This inhibitory effect of rapamycin appears to be selective for the p70\(s6k\) pathway because the activity of other insulin-stimulated protein kinases is not affected (25, 26).

A second insulin signaling pathway is initiated by the adapter protein growth factor receptor binding protein-2 (GRB-2). This protein also binds to phosphorylated IRS-1 through an SH2 domain interaction (8, 9). GRB-2 recruits the guanine nucleotide exchange factor mSOS to this complex; mSOS induces the formation of the active, GTP bound form of the oncogene p21\(ras\). The activation of p21\(ras\) leads to the induction of a cascade of activated protein kinases, namely, Raf-1, MAP kinase or ERK kinase (MEK), the p42/p44 isoforms of the mitogen-activated protein (MAP) kinase family of protein kinases and the p90 ribosomal S6 protein kinase (p90\(s6k\)) (27). The recent isolation of a selective inhibitor (PD98059) of MEK allows one to examine the role of p42/p44 MAP kinase in a number of insulin-regulated processes (28). This inhibitor blocks the stimulatory effects of insulin on MAP kinase, p90\(s6k\),...
and c-fos gene transcription in 3T3-L1 adipocytes or L6 myotubes (29).

GLUT4 is the major glucose transporter in fat and skeletal muscle and its translocation from an intracellular pool to the plasma membrane is stimulated by insulin (30, 31). Recently, inhibitors of insulin signaling pathways have been utilized to determine the mechanism by which insulin stimulates GLUT4 translocation. Wortmannin inhibits insulin-stimulated GLUT4 translocation and glucose transport in L6 myotubes and rat skeletal muscle (15, 16). While both rapamycin and wortmannin block the activation of p70S6K by insulin, only wortmannin inhibits the insulin effect on glucose transport and GLUT4 translocation in rat adipocytes and 3T3-L1 cells (17–20, 32). These data suggest that PI 3-kinase is necessary for the insulin-stimulated GLUT4 translocation and that p70S6K is not required. Therefore an alternative PI 3-kinase dependent pathway must be involved.

The coordinate regulation of GLUT4 and HKII is an essential component of glucose utilization in skeletal muscle and fat tissues (1, 2, 33). In view of this, it is of interest to compare the signaling pathway(s) utilized by insulin to regulate HKII and GLUT4. The data presented herein shows that both rapamycin and wortmannin inhibit insulin-induced HKII gene transcription, while PD98059 has no effect. Thus, the signaling pathway that leads to increased HKII gene transcription is distinct, at some point, from that utilized by insulin to regulate GLUT4 translocation.

MATERIALS AND METHODS

Tissue Culture—L6 cells, a myoblast cell line representative of rat skeletal muscle, were purchased from American Type Culture Collection (Rockville, MD) and were grown in 10-cm plates to near confluence. They were then induced to differentiate as described previously (4). The differentiated myotubes were maintained in Dulbecco’s modified Eagle’s medium (DMEM) containing 2% horse serum for 24 h before use.

Total RNA Extraction—L6 myotubes were incubated in serum-free DMEM for 24 h and then were placed in serum-free DMEM containing 100 nM insulin and/or the indicated agents for 3 h. Since the agents used were dissolved in dimethyl sulfoxide, an equal amount of dimethyl sulfoxide carrier was added to the cells with all treatments described herein. Total cellular RNA was extracted as described previously (5) or by using TRI Reagent according to the manufacturer’s protocol (Molecular Research Center, Inc., Cincinnati, OH). The RNA was dissolved in sterile H2O, and the concentration and purity of each sample was assessed by absorbance at 260 nm and by the 260 nm/280 nm ratio, respectively.

Ribonuclease Protection Assay—A linearized plasmid that contained a cDNA fragment for either rat HKI or HKII served as the template for the transcription of [α-32P]UTP-labeled antisense RNA probes (5). These probes were used to quantitate HKI or HKII mRNA in a ribonuclease protection assay, as described previously (5). The protected, labeled RNA was fractionated on a 5% polyacrylamide gel and the autoradiographs were quantitated by densitometry. Because of the use of different sized probes, HKI and HKII mRNAs could be quantitated in the same experiment.

Plasmid Construction—We have previously described the construction of chimeric fusion genes that contain various lengths of 5′-flanking sequence of the rat HKII gene ligated to a chloramphenical acetyltransferase (CAT) reporter gene.2 The largest of these constructs contains all 5.5 kilobase pairs of a 5′-flanking sequence cloned previously (4). The transgene used in the present study contains both the proximal 487 base pairs of 5′-flanking sequence and the initial 147 base pairs of 5′-untranslated sequence. This pHKIIICAT construct (−487/−147) contains all of the cis-acting regulatory sequence(s) necessary for mediating the effect of insulin on HKII gene transcription.3

Transfection and CAT Assay—L6 myotubes were transiently transfected using poly-l-ornithine and dimethyl sulfoxide shock as described previously (34). Following incubation with serum-free DMEM for 18 h, the cells were placed in serum-free DMEM containing the agents indicated for an additional 24 h. Cell extracts were prepared, and CAT assays were performed as described elsewhere (34). CAT activity in each extract was normalized for protein content, as measured by the Bio-Rad method (Bio-Rad).

MAP Kinase Activity—L6 myotubes were incubated in serum-free DMEM for 24 h and then were placed in serum-free DMEM containing 100 nM insulin and/or 10 μM PD98059 (a generous gift of Dr. A. R. Saltiel, Parke-Davis Pharmaceutical Research) for 15 min. The cells were washed with phosphate-buffered saline and harvested as described previously (13). MAP kinase activity was determined using myelin basic protein as a substrate as described elsewhere (35), except that the incubation was conducted for 10 min in the absence of okadaic acid. One unit of MAP kinase activity catalyzes the phosphorylation of 1 nmol of substrate/min.

Statistical Analysis—All data are presented as the mean ± the standard error of the mean (mean ± S.E.). Statistical comparisons were made by single factor factorial analysis of variance using STATVIEW II™ software for Apple Macintosh computers (Abacus Concepts, Berkeley, CA).

RESULTS

Effect of Wortmannin on Basal and Insulin-induced HKII mRNA—Insulin treatment of L6 myotubes for 3 h increased HKII mRNA by 2.2-fold (Fig. 1). This effect of insulin was blocked by wortmannin in an apparent dose-dependent manner. Wortmannin did not affect the basal amount of HKII mRNA. HKI mRNA was not affected by either insulin or wortmannin, thus wortmannin selectively prevented the stimulation of HKII mRNA by insulin. We have previously shown that insulin increases the rate of HKII gene transcription in L6 myotubes (4), and insulin does not affect HKII mRNA stability (data not shown). Thus, although HKII gene transcription was not directly measured in this study, we presume that changes in HKII mRNA reflect alterations in the rate of transcription of the gene. Accordingly, these findings suggest that PI 3-kinase

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2 Osawa, H., Robey, R. B., Printz, R. L., and Granner, D. K. (1996) J. Biol. Chem., in press.

3 R. B. Robey, H. Osawa, R. L. Printz, and D. K. Granner, unpublished observations.
may be involved in the regulation of HKII gene transcription.

Effect of Rapamycin on Basal and Insulin-induced HKII Gene Expression—Insulin activates ribosomal protein kinase, p70S6K, and evidence suggests that this action of insulin requires PI 3-kinase (32, 36). Rapamycin prevents the activation of p70S6K by insulin without affecting PI 3-kinase activity (22, 23). Therefore, rapamycin was used to determine whether p70S6K is also involved in HKII gene induction by insulin (Fig. 2). Rapamycin resulted in a concentration dependent reduction of the action of insulin without affecting basal HKII mRNA, even at the 100 nM concentration (Fig. 2, A and B). HKI mRNA was not affected by either insulin or rapamycin. Therefore, as with wortmannin, rapamycin selectively prevented the insulin-induced increase of HKII mRNA.

We have recently observed that insulin stimulates expression of HKII-CAT chimeric constructs that are transfected into L6 myotubes.3 The pHKIIICAT construct, which contains the HKII sequence from −487/+147 relative to the transcription initiation site, was therefore transfected into L6 myotubes and then insulin, with or without rapamycin, was added to the culture medium. Insulin resulted in a 3.5-fold increase in CAT expression (Fig. 2C), an effect that was almost completely blocked by 10 nM rapamycin. This concentration of rapamycin also showed a slight inhibitory effect on expression from the HKII promoter construct in the absence of insulin. These results suggest that the HKII promoter sequence within the pHKIIICAT construct responds to insulin, and that this response is dependent upon the activation of p70S6K.

Effect of PD98059 on Basal and Insulin-induced HKII Gene Expression—The protein kinase MEK directly phosphorylates and activates MAP kinase. Thus the activation of MAP kinase by insulin was used to determine the efficacy of the MEK inhibitor, PD98059, in L6 myotubes. Insulin (100 nM) increased MAP kinase activity by 1.6-fold, and PD98059 (10 µM) completely inhibited this effect (Table I). This concentration of PD98059 was used to test whether the inhibition of the Ras-MAP kinase pathway affects insulin-induced HKII gene expression (Fig. 3). Insulin increased HKII mRNA by ~2-fold without significantly changing HKI mRNA (Fig. 3, A and B). PD98059 had no effect on either basal or insulin-induced HKII mRNA and similarly did not affect HKI mRNA. In addition, in transfection experiments, PD98059 did not affect either basal or insulin-induced HKII promoter-CAT gene expression (Fig. 3C). These findings suggest that the Ras-MAP kinase pathway does not play a role in HKII gene induction by insulin.


discussion

Molecules involved in the insulin signal transduction pathway(s) are being characterized at a rapid pace. In spite of this progress, it is still not possible to describe a signal transduction pathway that accounts for the coordinated events involved in a complex process such as glucose utilization in tissues like fat and skeletal muscle. The initial events in glucose utilization involve glucose entry into the cell through a transporter like GLUT4, and glucose phosphorylation by a hexokinase, such as HKII (1, 2). Insulin promotes the translocation of the GLUT4 transporter and stimulates the rate of transcription of the HKII gene (4), but it is not clear whether these different processes are affected by the same signal transduction pathway. Similarly, it is of interest to know whether the signal transduction pathway used to stimulate transcription of the HKII gene is the same as that involved in the regulation of other insulin-regulated genes. Various inhibitors of key steps thought to be required in insulin signaling have been used to address these questions.

Wortmannin has been employed to predict a role for PI 3-kinase in the regulation of phosphoenolpyruvate carboxyki-
nase (PEPCK) gene transcription by insulin (21), the antilipo-
lytic action of insulin in adipocytes (12), the inhibition of gly-
cogen synthase kinase-3 by insulin (13), and insulin-induced
free glucose uptake (see Refs. 14–20 and references therein) and
glycogen synthesis (17, 23). The fact that wortmannin prevents
the insulin-stimulated accumulation of HKII mRNA in L6 myo-
tubes suggests that there is a requirement for this lipid kinase
in the regulation of HKII gene transcription (see Fig. 1), since
HKII mRNA is increased by insulin primarily by an increase in
gene transcription (Fig. 1) (4). p70\textsuperscript{S6K} is assumed to lie down-
stream of PI 3-kinase in an insulin signaling pathway (Fig. 4).
Since rapamycin also inhibits the regulation of HKII gene
expression by insulin (Fig. 2), a PI 3-kinase/p70\textsuperscript{S6K}-dependent
pathway appears to be important for this process (Fig. 4). This
is in contrast to the regulation of GLUT4 translocation by
insulin, which is not affected by rapamycin (18). Therefore,
insulin appears to employ distinct signaling pathways down-
stream of PI 3-kinase to regulate these two events involved in
glucose utilization. Different signaling pathways are also in-
volved in the regulation of the HKII and PEPCK genes by
insulin as wortmannin prevents the effect of insulin on both
genes, whereas rapamycin blocks the action of insulin on the
HKII gene, but has no effect on the PEPCK gene (Fig. 2) (21).
Interestingly, wortmannin also prevents the stimulation of
the Ras-MAP kinase pathway by insulin in L6 myotubes (13).
Activation of this pathway is also blocked by PD98059, a selec-
tive inhibitor of MEK (28, 29). The Ras-MAP kinase pathway,
which has been implicated in the regulation of c-fos gene tran-
scription by a variety of agents, including insulin (29), does not
appear to be required for insulin-mediated regulation of glu-
cose transport, lipogenesis, PEPCK gene transcription, or gly-
cogen synthesis (21, 29, 37). Although PD98059 clearly blocked
insulin stimulation of the p42/p44 isoforms of MAP kinase in
the L6 myotubes (Table I), it had no effect on the regulation of
HKII gene transcription by insulin (Fig. 3).

Non-insulin-dependent diabetes mellitus (NIDDM) is char-
acterized by insulin resistance and impaired insulin secretion
(33). Glucose utilization is decreased in skeletal muscle of per-
son with NIDDM, and glycogen synthesis and glucose-6-phos-
phate are also decreased (38, 39), all of which could be attrib-
uted to decreased rates of glucose phosphorylation. Thus,
faulty regulation of HKII by insulin could be a component of the
metabolic defect seen in NIDDM. Insulin receptor phosphoryl-
ation, IRS-1 phosphorylation, and PI 3-kinase activity are de-
creased in skeletal muscle in insulin-resistant obese subjects
(40), and p70\textsuperscript{S6K} activity is reduced in skeletal muscle of insu-
lin-resistant Pima Indians (41). These findings suggest that
decreased HKII gene expression that results from a faulty
signaling pathway leading to p70\textsuperscript{S6K} might be involved in insu-
lin resistance and NIDDM. Indeed, HKII gene expression is
impaired in skeletal muscle isolated from persons with NIDDM
(42).

In summary, a PI 3-kinase p70\textsuperscript{S6K}-dependent pathway is
required for the regulation of HKII gene transcription by insu-
lin. Two different pathways downstream from PI 3-kinase ap-
pear to be involved in insulin-regulated glucose utilization: 1) a
rapamycin-insensitive pathway that leads to GLUT4 translo-
cation, and 2) a rapamycin-sensitive pathway that leads to
HKII gene transcription (Fig. 4). The role these pathways play

![Fig. 3. Effect of PD98059 on basal and insulin-induced HKII gene expression.](image1)

![Fig. 4. Possible insulin signaling pathways leading to HKII and PEPCK gene transcription and GLUT4 translocation.](image2)
in the decreased peripheral glucose utilization that is characteristic of NIDDM merits further investigation.

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