Insulin Receptor at the Mouse Hepatocyte Nucleus after a Glucose Meal Induces Dephosphorylation of a 30-kDa Transcription Factor and a Concomitant Increase in Malic Enzyme Gene Expression[1]

Nana Gletsu,*† W. Dixon† and M. T. Clandinin*†**[2]

*Nutrition and Metabolism Research Group, †Department of Agricultural, Food and Nutritional Science, **Department of Medicine, University of Alberta, Edmonton, AB, Canada T6G 2P5

ABSTRACT Insulin receptor translocation to the nucleus may represent a mechanism for activation of transcription factors controlling lipogenic gene expression in the mouse hepatocyte. Insulin stimulation was achieved in vivo by oral glucose feeding of mice deprived of food for 24 h. Hepatocytes were fractionated after the glucose meal and nuclei were purified. Insulin receptor levels and phosphorylation state in nuclei were assessed by immunoassay. Insulin receptor significantly increased from basal levels in hepatocyte nuclei within 15 min of the glucose meal. Immunoassay using antiphosphotyrosine indicated that phosphorylation of nuclear insulin receptor increased, whereas phosphorylation of a 30-kDa DNA-binding protein significantly decreased within 15 min of the glucose meal. Glucose treatment significantly increased expression of malic enzyme within the time frame of insulin receptor translocation to the nucleus. Nuclear protein binding to an insulin response element (IRE) within the malic enzyme gene promoter significantly increased within 15 min of the glucose meal. When cell nuclei were isolated from mice that had been deprived of food and treated in vitro with purified, activated insulin receptor, changes were observed in DNA-binding protein phosphorylation and IRE-binding in the absence of cytoplasmic insulin signaling. In vitro incubation of nuclei with activated insulin receptor significantly decreased phosphorylation of a 30-kDa DNA-binding protein compared with basal levels. Increased binding of nuclear proteins to malic enzyme IRE was observed upon stimulation of isolated nuclei with activated insulin receptor. These results suggest that nuclear insulin receptors induce malic enzyme gene expression by regulating phosphorylation of IRE transcription factors. J. Nutr. 129: 2154–2161, 1999.

KEY WORDS: • *insulin receptor* • *hepatocyte* • *mice* • *lipogenesis*

Insulin binding and insulin receptor (IR)[3] tyrosine kinase activation occur at the cell surface (Kasuga et al. 1982). Insulin signaling within the cell begins with IR tyrosine kinase activation of its most immediate substrate, insulin receptor substrate (IRS-1) (Sun et al. 1991). The activity of IRS-1 is thought to be responsible for the transmission of the insulin signal to other cytosolic mediators, including phosphatidylinositol 3′-kinase (P-I-3′K) and mitogen-activated protein kinase (MAP-K) within the cell (Kahn et al. 1993). Gene regulation by insulin is maintained in animals deficient in IRS-1 (Araki et al. 1994, Tamemoto et al. 1994), which suggests the existence of other signaling pathways that by-pass IRS-1. Furthermore, for phosphoenolpyruvate carboxykinase (PEPCK) and hexokinase genes, inactivation of signal transduction via MAP-K and P-I-3′K does not abolish transcription responsiveness to insulin (Gabbay et al. 1996, Osawa et al. 1996). Thus, insulin signal transduction to the nucleus may depend on alternate pathways that are independent of IRS-1, MAP-K and P-I-3′K activation.

There is evidence that insulin (Kim and Kahn 1993, Podlecki et al. 1987, Smith and Jarett 1987) and other polypeptide hormones (Fraser and Harvey 1992, Jiang and Schindler 1990, Lobie et al. 1994) activate nuclear processes by translocation of activated receptors to the nucleus. Insulin receptors are translocated to the nucleus via an internalization process that begins upon ligand binding to receptors on the cell membrane (Kim and Kahn 1993, Podlecki et al. 1987, Smith and Jarett 1987). A newly translocated IR retains its tyrosine kinase activity (Kim and Kahn 1993), which suggests that it is able to phosphorylate tyrosine sites on nuclear proteins. Accumulation of the IR in the nucleus occurs after insulin stimulation in vivo (Gletsu et al., in press). The objective of this study was to determine whether changes in nuclear protein phosphorylation and gene expression could be induced by insulin stimulation within the time frame of translocation of IR to the hepatocyte nucleus. To test for this mode of insulin action, mice were food deprived for 24 h and then given a meal of glucose to produce insulin stimulation. Hepatocyte nuclei were isolated at selected time points after the

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[2] To whom correspondence should be addressed.

[3] anti-PY, anti-phosphotyrosine; FAS, fatty acid synthesis; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; IR, insulin receptor; IRE, insulin responsive elements; IRS-1, insulin receptor substrate; MAP-K, mitogen-activated protein kinase; ME, malic enzyme; PEPCK, phosphoenolpyruvate carboxykinase; P-I-3′K, phosphatidylinositol 3′-kinase; PMSF, phenylmethylsulfonyl fluoride; TIU, trypsan inhibitory units.

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glucose meal and were assayed for IR levels, IR autophosphorylation and the transcription rate of insulin-responsive genes involved in lipogenesis.

Although IR accumulates in the nucleus of glucose-treated mice (Gletsu et al., in press), changes that occur in nuclear processes may be a result of cytoplasmic signaling initiated by IR activation of IRS-1. To determine whether changes in nuclear protein phosphorylation and gene expression after in vivo stimulation with insulin could be produced in the absence of cytoplasmic signaling, nuclei taken from hepatocytes of food-deprived mice were incubated with purified IR. The effect of this incubation of IR on the phosphorylation of nuclear proteins and the affinity of malic enzyme insulin responsive elements (IRE)-binding proteins was determined.

MATERIALS AND METHODS

Animals care and treatments. Male C57 Black 6 mice (Jackson Laboratories, Bar Harbor, ME), were housed in groups of four to six in polypropylene cages in a humidity- and temperature-controlled room with a 12-h light:dark cycle. Mice were given free access to a nonpurified diet from the age of weaning (Laboratory Standard Rodent Diet 5001, PMI Feeds, Brentwood, MO). At 8 wk of age, mice were deprived of food for 24 h beginning at 0900 h on the day before the experimental day. Mice treated with glucose were then given an oral gavage of 3 g glucose/kg body weight. At various times after the gavage, mice were anesthetized with halothane, and blood was removed by cardiac puncture for glucose and insulin determinations. Mice were killed by cervical dislocation. Procedures were in accord with the NIH guidelines (NRC 1985) and approved by an animal ethics committee.

Nuclei isolation. Liver was immediately excised and minced in homogenization buffer containing 250 mmol/L glucose, 50 mmol/L Tris-HCl, 1 mmol/L benzamidine-HCl, 1 mmol/L phenylmethylsulfonyl flouride (PMSF), 10 μmol/L sodium orthovanadate, 1 mmol/L $MgCl_2$, 2.1 μmol/L leupeptin, 1.5 μmol/L pepstatin, 4.4 trypsin inhibitory units (TIU)/L aprotinin (pH 7.5). Nuclei were isolated as described by Blobel and Potter (1966). Final nuclei pellets were suspended in nuclei isolation buffer containing 50 mmol/L Tris-HCl, pH 7.5, 250 mmol/L sucrose, 5 mmol/L $MgCl_2$, 2.5 mmol/L KCl, 1 mmol/L benzamidine-HCl, 1 mmol/L PMSF, 10 μmol/L sodium orthovanadate, 2.1 μmol/L leupeptin, 1.5 μmol/L pepstatin and 4.4 TIU/L aprotinin (pH 7.5). To check purity of isolated nuclei, nuclei suspensions were inspected under the light microscope after staining with 0.2% (v/v) methylene blue and ethidium bromide (100 mg/L) at 400X magnification. Cell nuclei were assayed for 5′nucleotidase activity as a measure of plasma membrane contamination (Aronson and Touster 1974), succinate cytochrome c reductase activity as a measure of mitochondrial contamination (Green et al. 1955) and glucose-6-phosphatase activity as a measure of endoplasmic reticulum (Swanson, 1955).

Immunoblotting. A polyclonal antibody to purified IR was raised using standard techniques (Assil et al. 1992) in Flemish Giant X Dutch Lop Ear rabbits, housed at the Biological Sciences Center at the University of Alberta. Antibody was purified from immune rabbit serum by incubation of serum with IR blotted on nitrocellulose paper. Purified antibody, referred to as anti-IR, was tested for effectiveness in immunoassays and was found to detect IR preparations (produced both commercially and in the laboratory) in immunoblotting and to immunoprecipitate IR in plasma membrane and nuclei preparations as effectively as commercially obtained antibodies including anti-human IR α-subunit GRO7 from Oncogene Science (Uniondale, NY) or anti-human β-subunit from Transduction Laboratories (Lexington, KY).

Samples of rat liver nuclei extract (100 μg protein) were precipitated by adding an equal volume of cold acetone, incubated at −20°C for 60 min and then centrifuged at 5000 × *g* to pellet proteins. Samples were subjected to SDS-PAGE using a 7.5% (wt/v) gel under reducing conditions (Laemmli 1970) and electrotransferred to nitrocellulose. Nitrocellulose sheets were blocked for 1 h at 25°C with 50 g/L bovine serum albumin Fraction V (Sigma Chemical, St. Louis, MO) for analysis using anti-IR or 30 g/L nonfat skim milk in PBS (pH 7.3) for analysis using antiphosphotyrosine (anti-PY), (Upstate Biotechnology, Lake Placid, NY). Nitrocellulose sheets were then probed with anti-IR (1/25 dilution) or anti-PY (1 mg/L) overnight at 4°C in blocking solution containing 0.1% (v/v) Tween 20 and washed with PBS, 0.1% (v/v) Tween 20. Detection was with horseradish peroxidase-conjugated secondary antibody (1/3000 dilution), followed by enhanced chemiluminesce imaging on X-ray film using ECL reagent (Amersham, Little Chafont Buckinghamshire, UK). The intensities of the bands were quantified by scanning densitometry using the GS-670 Imaging Densitomer (BioRad Laboratories, Hercules, CA).

Immunoprecipitation. Nuclear extract (300 μg protein) was precleared three times each with rabbit serum agarose (Sigma Chemical) and then with recombinant Protein A-agarose (Upstate Biotechnology) and incubated with 5 μg anti-IR overnight at 4°C. Recombinant Protein A-agarose [100 μL of a 50% (v/v) suspension] was added to the mixture and incubated for 2 h at 4°C with rotation. Samples were then centrifuged for 1 min at 14,000 × *g*, and the pellet containing anti-IR-antigen-agarose conjugate was washed three times in PBS (pH 7.3) buffer supplemented with 1% (v/v) Triton X-100, 5 g/L sodium deoxycholate, 1 g/L SDS, 0.04 mg/L NaF, and then twice with PBS, 0.04 mg/L NaF. The washed beads were resuspended in 50 μL of Laemmli sample buffer with 5% (v/v) 2-mercaptoethanol, boiled for 5 min, and centrifuged for 5 min at 14,000 × *g*; the supernatant was then analyzed on 7.5% (wt/v) SDS-PAGE followed by Western blotting with anti-PY.

Phosphosphorylation of nuclear proteins binding to DNA. DNA-binding proteins were prepared from nuclei extract as described (Jaumot et al. 1996). The purified nuclei pellet was resuspended in buffer containing 50 mmol/L Tris-HCl, pH 7.4, 250 mmol/L sucrose, 5 mmol/L $MgSO_4$, 1 mmol/L PMSF, 1.5 TIU/L aprotinin, and 5 μmol/L leupeptin. DNase 1 and RNase A were added to suspensions to 250 g/L final concentration each. After 1 h of incubation, the nuclei were sedimented at 5000 × *g* for 10 min. The supernatant, containing soluble nuclear proteins and weakly DNA-associated proteins, was collected. The pellet was resuspended in a buffer containing 10 mmol/L Tris-HCl, pH 7.4, 0.2 mmol/L $MgSO_4$, 1 mmol/L PMSF, and 1.5 TIU/L aprotinin. This buffer, which contained 2.0 mol/L NaCl, was then slowly added to preparations to a final NaCl concentration of 1.6 mol/L. After 15 min incubation, the residual structures were sedimented at 5000 × *g* for 20 min. The supernatant, which contained tightly bound DNA proteins, was collected and stored at −70°C. To determine tyrosine phosphorylation state, 100 μg of DNA binding proteins from each sample was subjected to SDS-PAGE using a 10% (wt/v) polyacrylamide gel. DNA-binding proteins were analyzed by Western blotting using anti-PY as described above.

Phosphorylation of isolated DNA-binding proteins by purified IR. IR was purified from plasma membrane as described (Fujita-Yamaguchi et al. 1983, Helmerhorst and Nakhoul 1993) and assayed during various steps in the purification with the use of insulin binding assays (Fujita-Yamaguchi et al. 1983) and Western blotting assays using anti-IR antibody (Transduction Laboratories, Lexington, KY). Purified IR was stored in 50% (v/v) glycerol at −70°C.

Isolated DNA-binding proteins were prepared from the nuclei of hepatocytes taken from mice that were deprived of food for 24 h. Protein (50 μg) from each sample was incubated with reaction mixture containing 60 mmol/L Tris-HCl, pH 7.5, 10 mmol/L $MgCl_2$, 230 mmol/L NaCl, 1 mmol/L PMSF, 0.4 mmol/L adenosine triphosphate, 8 mmol/L $MnCl_2$, 2 mmol/L dithiothreitol and 4.2 μmol/L leupeptin. To begin the labeling of DNA binding proteins, [γ^{32}P]-ATP (74 kBq) was added and the mixture incubated for 15 min at room temperature. At the same time, 2 μg purified IR preparation in reaction mixture without dithiothreitol was activated using 10 nmol/L insulin for 15 min at room temperature. To the labeled DNA binding proteins that had been labeled with [γ^{32}P]-ATP, 0.2 μg of activated receptor was added. The reaction was conducted for 0, 10, 15 and 60 min, stopped by adding 4X Laemmli sample buffer and then boiled for 5 min. Controls for each time were prepared by incubating an equivalent DNA-binding protein extract for the same amount of time with [γ^{32}P]-ATP but without the addition of activated IR.

Samples were loaded onto a 7.5% (wt/v) polyacrylamide gel and subjected to SDS-PAGE at 25 mA. The gel was stained with 0.25 g/L Coomassie brilliant blue R250 (BioRad), 40% (v/v) methanol and 10% (v/v) acetic acid, and vacuum dried. The ^{32}P-labeled DNA binding proteins were visualized by autoradiography and quantitated by densitometry using the GS-670 Imaging Densitometer (BioRad).

Gel shift assay. Nuclear extracts were prepared from liver as described above with the exception that final pellets were suspended in 25 mmol/L HEPES, pH 7.6, 40 mmol/L KCl, 0.1 mmol/L EDTA, 1 mmol/L dithiothreitol and 10% (v/v) glycerol, and stored at −70°C until use. Gel mobility assays were performed as described (Chodosh et al. 1988). The oligonucleotides used in the assay (5′-CCCGCCCCGCCTCCTCGCA-3′ and its complement) corresponded to the sequences −175 to −156 of the promoter region of the malic enzyme gene. The underlined sequence was described previously as an IRE within the malic enzyme gene promoter (Garcia-Jimenez et al. 1994). The oligonucleotides (50 μg each) were labeled by incubating the oligonucleotides with 5.55 MBq of [γ-^{32}P]-ATP, and T4 polynucleotide kinase (10 U) (GIBCO BRL Life Technologies Gaithersburg, MD) for 1 h at 37°C. Labeled DNA was purified by adding 0.2 vol of 5 mol/L ammonium acetate and 2 vol of ethanol. The DNA-labeled pellet was collected by centrifugation at 14,000 × *g* for 15 min (Jouan Microcentrifuge, Winchester VA) and dissolved in 50 μL water; 2 pmol of labeled probe (333 Bq) was used per reaction.

The binding reaction was conducted with 5 μg of nuclear extract in a binding reaction mixture containing 40 mmol/L HEPES, pH 7.9, 200 mmol/L KCl, 0.5 mmol/L dithiothreitol, 0.2 mmol/L EDTA, 10% (v/v) glycerol and 3 μg Sonicated Calf Thymus DNA (Pharmacia, Piscataway, NJ), and was incubated for 15 min on ice. In competition experiments, unlabeled oligonucleotides were added in excess (160 times) or 30 ng of EBNA-1 DNA, an 82-mer unrelated DNA, was added (Pharmacia). A labeled probe (2 pmol) was added to the mixture and incubated for 30 min at room temperature. Loading dye (2 μL) containing 250 mmol/L Tris-HCl (pH 7.5), 2.0 g/L bromophenol blue, 2.0 g/L xylene cyanol and 40% (v/v) glycerol was added to the mixture. Free DNA and DNA-protein complexes were resolved on native 5% (wt/v) polyacrylamide gels in 0.5X TBE (1X TBE is 90 mmol/L Tris, 90 mmol/L boric acid and 1 mmol/L EDTA, pH 8.0), run at 20 mA before being vacuum dried and exposed to an X-ray film at −70°C. Bands were quantified using the GS-670 Imaging Densitomer from BioRad.

Incubation of nuclei with purified IR before gel shift assay. Experiments were designed to evaluate nuclear protein binding affinity to malic enzyme (ME)-IRE after incubation for varying lengths of time with purified IR. Activated IR (0.25 μg) was added to nuclear extracts for 10, 15 or 30 min incubation at room temperature. Nuclear extracts were then incubated in binding mixture containing 40 mmol/L HEPES, pH 7.9, 200 mmol/L KCl, 0.5 mmol/L dithiothreitol, 0.2 mmol/L EDTA, 10% (v/v) glycerol and 3 μg Sonicated Calf Thymus DNA, for 15 min on ice. The gel shift assay was continued as described above.

Experiments were designed to evaluate nuclear protein binding affinity to ME-IRE after incubation with varying doses of purified IR. Activated IR was incubated with nuclear extracts for 15 min at room temperature. Bovine serum albumin (Fraction V, Sigma Chemical) was substituted for IR so that the protein added to nuclear extracts totaled 0.05 mg/L. IR without insulin stimulation was added to the incubation mixture and was used in place of activated IR to serve as a control. Nuclear extracts were then incubated with oligonucleotides in binding mixture and gel shift assay continued as described above.

Northern blot analysis. Liver taken from 8-wk-old mice, treated as described above, was extracted at selected times after oral glucose gavage, immediately frozen in liquid nitrogen and stored at −70°C. Total RNA was extracted from tissue using the guanidium isothiocyanate, phenol/chloroform method (Chomczynski and Sacchi 1987) using a commercially available TRIZOL reagent (GIBCO BRL). The quantity and purity of RNA were determined by absorbance at 260 and 280 nm. RNA (30 μg) was analyzed by Northern blotting (Seed 1979) after electrophoresis on a 1% (wt/v) agarose/0.66 mol/L formaldehyde gel and transferred onto a nitrocellulose membrane (Micron Separation, Westborough, MA). For each blot, equal loading of lanes and integrity of RNA were confirmed by ethidium bromide fluorescence of ribosomal 18S and 28S RNA.

The cDNA encoding for malic enzyme was provided by Dr. D.W. Back, (Queen's University, Kingston, ON, Canada). The cDNA encoding for fatty acid synthase (FAS) was provided by Dr. S. Smith, (Children's Hospital Oakland Research Institute, Oakland, CA). Probes were labeled with [α-^{32}P]-dATP using the Random Primer Labeling System (GIBCO BRL).

Statistical evaluation. For each analysis, three experiments were conducted, each on different days. Three different mice were examined for each time point selected in each of three experiments (n = 9). For the effects of oral glucose gavage on IRE-binding, n = 6 for the 0- and 15-min times and n = 2 for the 180-min time after oral glucose gavage. For the effects of IR incubation on IRE-binding, five mice were used for each dose of IR assessed, and one mouse was used for the time-response experiment. The difference between selected time points after oral glucose was assessed by using ANOVA procedures (SAS Institute, Cary, NC). A significant difference between time points was determined by Duncan's multiple range test (Duncan 1955, Steel and Torrie 1980) ($P < 0.05$). In some cases as indicated, differences between comparisons were assessed using Student's t tests ($P < 0.05$).

TABLE 1

Serum glucose and insulin in food-deprived mice at selected times after oral glucose gavage[1,2]

Time	Serum glucose	Serum insulin
min	*mmol/L*	*pmol/L*
Basal	6.1 ± 0.2[a]	20.3 ± 0.6[a]
10	26.9 ± 1.1[d]	28.7 ± 1.2[b]
15	23.5 ± 0.7[c]	29.5 ± 1.0[b]
180	8.9 ± 0.5[b]	27.3 ± 1.8[b]

[1] Eight-week old mice were given an oral glucose gavage of 3 g/kg body weight. At selected time points after gavage, mice were anesthetized and blood was removed by cardiac puncture.

[2] Data represent means ± SEM, n = 9. Values in a column with different letters are significantly different ($P < 0.05$).

RESULTS

Animal characteristics. Body and liver weights of mice at 8 wk of age averaged 28.1 ± 0.4 and 1.18 ± 0.03 g, respectively. Body and liver weights did not differ among mice used for different treatments of oral glucose.

Recovery and purity of nuclei preparations. Recovery of nuclei estimated from DNA determinations was 6.6 ± 0.3% in nuclei preparations, which is a typical percentage for recovery of DNA after nuclei isolation from liver (Graham 1993). On the basis of the specific activity measured in the nuclei, divided by the specific activity in isolated plasma membrane, the amount of contamination of nuclei by plasma membrane was 3.8 ± 0.1%, the amount of contamination of nuclei by mitochondrial succinate cytochrome c reductase activity was typically <1% and the nuclei preparations exhibited 7.60 ± 1.26% contamination by endoplasmic reticulum.

Insulin and glucose determinations. Food-deprived mice were given an oral dose of glucose to induce insulin stimulation in vivo. At 10 and 15 min after this dose, serum glucose levels increased from basal levels ($P < 0.05$) (**Table 1**). By 180 min after the glucose gavage, glucose levels dropped, but were still significantly greater than basal levels. The oral dose of glucose induced a rise of serum insulin at 10 and 15 min compared with basal levels ($P < 0.05$) (Table 1). Serum insulin levels remained elevated compared with basal levels at

TABLE 2

Insulin receptor (IR) levels and autophosphorylation in the hepatocyte nucleus of food-deprived mice at selected times after oral glucose gavage[1,2]

Time	IR levels	β-subunit autophosphorylation
min	*densitometer units*	
Basal	6.2 ± 1.1[a]	9.2 ± 2.5[a]
15	10.9 ± 1.2[b]	22.7 ± 8.1[b]
180	17.3 ± 2.2[c]	29.8 ± 5.6[b]

[1] Eight-week old mice were given an oral glucose gavage of 3 g/kg body weight. At selected time points after gavage, mice were killed and liver nuclei isolated. IR levels were determined by Western blotting using anti-IR. Tyrosine phosphorylation of 95 kDA subunit of the IR was determined by immunoprecipitation of nuclei extracts using anti-IR and then by Western blotting using antiphosphotyrosine. Values are expressed in densitometer units.

[2] Data are means ± SEM, $n = 9$. Values in a column with different letters are significantly different ($P < 0.05$).

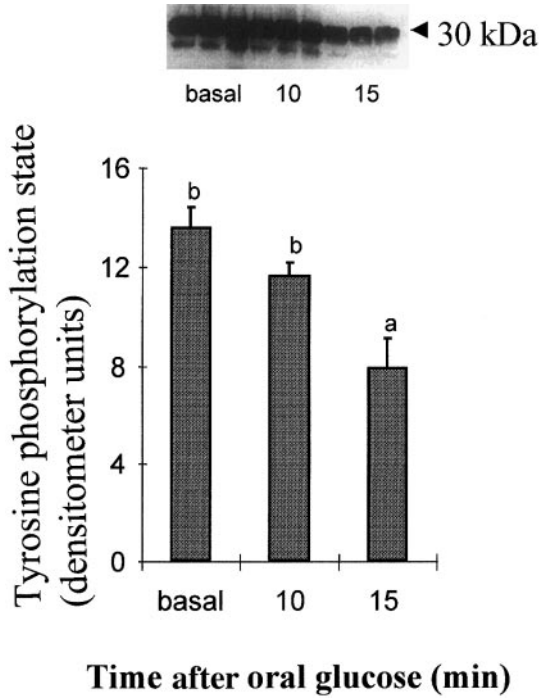

FIGURE 1 Tyrosine phosphorylation of DNA-binding proteins in the mouse hepatocyte nucleus at selected times after oral glucose gavage. Eight-wk-old mice were food deprived for 24 h and then given an oral glucose gavage of 3 g/kg body weight. At 0, 10 and 15 min after glucose treatment, mice were killed and liver nuclei isolated. DNA-binding proteins were prepared and their tyrosine phosphorylation determined by Western blotting with antiphosphotyrosine. Values are expressed in densitometer units. Data represents means ± SEM, $n = 9$. Values not sharing a common letter are significantly different ($P < 0.05$).

180 min after gavage. The increase in both serum glucose and insulin within 10 and 15 min after glucose gavage suggests that insulin stimulation of insulin-responsive tissues occurs in vivo within that time frame.

Nuclear IR levels. Anti-IR antibody recognized IR in 1% (v/v) Triton X-100–solublized nuclear extracts. Because Triton X-100 at 1% (v/v) is able to solubilize the outer but not the inner nuclear membrane, this suggests that the IR is associated with the outer nuclear membrane (Lobie et al. 1994). Western blotting analysis of nuclear extracts at selected time points after oral glucose using anti-IR antibody revealed that levels of IR increased by 81% at 15 min after glucose gavage compared with levels at basal conditions ($P < 0.05$) (**Table 2**). At 180 min, levels of IR in the nucleus increased 175% from basal conditions ($P < 0.05$) and were higher than IR levels found at 15 min ($P < 0.05$).

Nuclear IR autophosphorylation. Upon insulin binding to the α-subunit, the β-subunit of the IR undergoes tyrosine phosphorylation. Table 2 depicts the changes in phosphorylation state of the IR β-subunit in the hepatocyte nucleus after oral glucose gavage. In vivo stimulation increased receptor phosphorylation by 1.5-fold ($P < 0.05$) at 15 min and by 2.4-fold at 180 min ($P < 0.05$) compared with basal conditions.

Nuclear extract protein phosphorylation. Putative endogenous substrates of the nuclear IR tyrosine kinase were determined by assessing the in vivo tyrosine phosphorylation of DNA-binding proteins in the nucleus, using anti-PY, at selected time points after oral glucose gavage. A 31% decrease in tyrosine phosphorylation of a nuclear protein, ~30 kDa, was observed by 15 min after in vivo stimulation ($P < 0.05$) (**Fig. 1**).

Insulin stimulation of gene expression. The effect of in vivo insulin stimulation on expression of insulin responsive genes was assessed by determining the levels of mRNA transcripts of these genes detected in mouse liver samples after the oral glucose dose. FAS, ME and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA levels at selected times after glucose gavage were determined by Northern blot analysis using cDNA probes for the respective genes. At 15 min after oral glucose gavage, ME mRNA was increased by 35% ($P < 0.05$) compared with basal levels; however, by 180 min, ME mRNA levels were not different than basal levels (**Table 3**). GAPDH mRNA levels increased from basal by 16% at 15 min ($P < 0.05$) and then decreased at 180 min after glucose gavage to basal levels (Table 3). The level of FAS mRNA, however, did not differ from basal at either 15 or 180 min after insulin stimulation (data not shown).

Incorporation of P^{32} into DNA-binding proteins after incubation with IR. DNA-binding proteins prepared from isolated hepatocyte nuclei taken from mice that had been food deprived for 24 h were labeled with [γ^{32}P]-ATP for 15 min and

TABLE 3

Gene expression of malic enzyme and glyceraldehyde-3-phosphate at selected times after oral glucose gavage[1,2]

Time	mRNA levels of malic enzyme	mRNA levels of glyceraldehyde-3-phosphate dehydrogenase
min	*densitometer units*	
Basal	0.84 ± 0.05[a]	0.91 ± 0.03[a]
15	1.12 ± 0.05[b]	1.05 ± 0.04[b]
180	0.97 ± 0.07[a,b]	1.87 ± 0.05[a,b]

[1] Eight-week old mice were given an oral glucose gavage of 3 g/kg body weight. At selected time points after gavage, mice were killed and liver extracted. mRNA levels of malic enzyme and glyceraldehyde-3-phosphate were determined by Northern blotting. Values are expressed in densitometer units.

[2] Data are means ± SEM, $n = 9$. Values in a column with different letters are significantly different ($P < 0.05$).

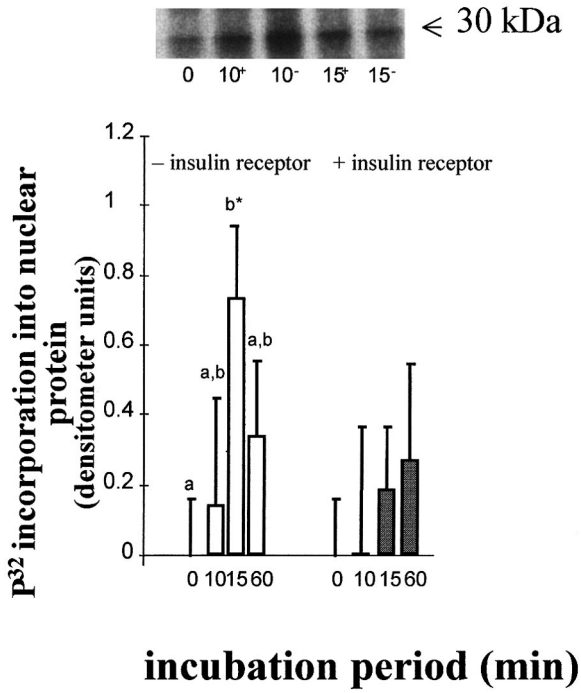

FIGURE 2 Incorporation of ^{32}P-phosphate into a 30-kDa nuclear protein after incubation of mouse hepatocyte nuclei with insulin receptor. Nuclei were isolated from hepatocytes taken from mice that were food deprived for 24 h. DNA-binding proteins were prepared from nuclei. Proteins were labeled with [γ-^{32}P]-ATP for 20 min before the addition of 0.2 μg of activated insulin receptor (IR). The reaction was conducted for 0, 10, 15 and 60 min and stopped by adding sample buffer and boiling for 5 min. Controls for each time contained no IR. Proteins were subjected to SDS-PAGE followed by autoradiography to detect radioactivity incorporated into protein. Bands were quantitated by densitometry and values are expressed as a change from basal in densitometer units (0 = 0.95 ± SEM, n = 9. Comparisons of the effect of time within each treatment with (+) or without (−) the addition of IR were assessed by ANOVA. Values not sharing a common letter are significantly different. Comparisons of the effect of treatment for each time were made using Student's t test ($P < 0.05$). The value labeled with (*), obtained at 15 min without IR, is significantly different from the value obtained at 15 min with IR.

then incubated for a period of time in the presence or absence of purified IR. The amount of labeled phosphate incorporated into several DNA-binding proteins was observed to increase with time with or without IR. IR added to nuclear proteins decreased the incorporation of ^{32}P-phosphate into a DNA-binding protein that migrated at 30 kDa (**Fig. 2**) on SDS polyacrylamide gels. At 15 min of incubation with IR, the amount of ^{32}P-phosphate observed in p30 was reduced by 73% compared with the levels of ^{32}P-phosphate observed in the absence of IR.

Transcription factor binding to ME-IRE. The effect of oral glucose administration on the binding affinity of proteins isolated from hepatocyte nuclei to ME-IRE was determined. Consistent with another report (Garcia-Jimenez et al. 1994), results from gel-shift assays suggest that two nuclear proteins bind to the ME-IRE. The binding of nuclear proteins to the labeled IRE probe was specific; it was abolished in the presence of excess unlabeled ME-IRE but was retained in the presence of an unrelated oligonucleotide. Compared with nuclear protein binding to the IRE within the malic enzyme gene at basal conditions, binding increased within 15 min after glucose treatment and decreased by 180 min after oral glucose gavage ($P < 0.05$) (**Fig. 3**).

Transcription factor binding to ME-IRE after incubation with IR. The effect of IR on the binding affinity of proteins to the malic enzyme promoter was assessed by incubating purified IR with preparations of nuclear proteins isolated from the liver of food-deprived mice. A time-course experiment demonstrated that incubation of IR with nuclear preparations before the addition of labeled probe increased the protein-DNA complex over that observed in the absence of IR (data not shown). Increasing doses of IR were added to nuclear preparations isolated from the liver of food-deprived mice before the incubation of nuclear proteins with the labeled probe. The complex formed between the labeled IRE probe and proteins present in the nuclear preparations was increased with increasing amounts of IR added (**Fig. 4**). Dose-response studies demonstrated that effects of IR were half-maximal at a dose of 0.0063 g/L and maximal at ~0.025 g/L.

DISCUSSION

This study demonstrates that IR accumulates in the hepatocyte nucleus after an increase in serum glucose and insulin levels induced by oral glucose administration. An increase in IR levels and autophosphorylation was detected in nuclei fractionated to purity at 15 and 180 min after an oral dose of glucose. Within the time course of active IR accumulation in the cell nucleus, the dephosphorylation of a nuclear DNA-binding phosphoprotein and the transient increase in gene

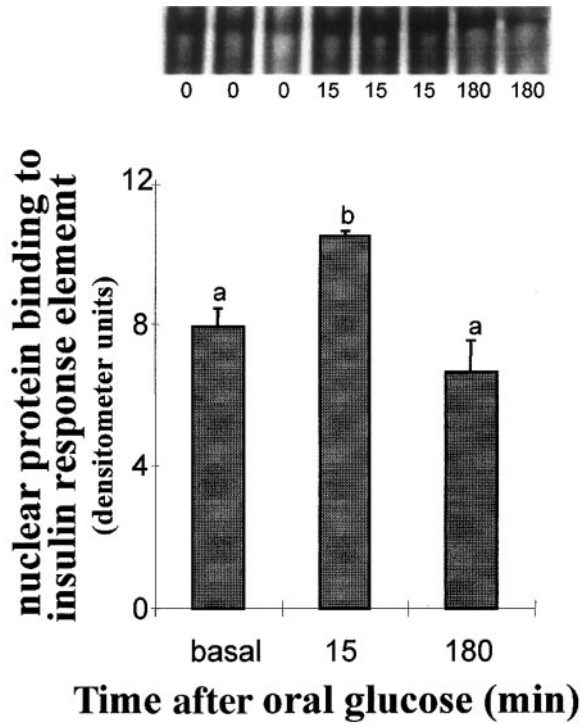

FIGURE 3 Mouse hepatocyte nuclear protein binding to malic enzyme-insulin response element (ME-IRE) in food-deprived mice after oral glucose treatment. Mice were food deprived for 24 h and then given an oral glucose gavage of 3 g/kg body weight. Mice were killed at 0, 15 and 180 min after oral glucose administration. Liver nuclei were isolated, and transcription factor binding to the ^{32}P-labeled ME-IRE probe assessed by gel shift assay. Bands were quantitated by densitometry and values are expressed in densitometer units. The effect of time after oral glucose treatment on ME-IRE binding was assessed by ANOVA. Values not sharing a common letter are significantly different ($P < 0.05$). Data represents means ± SEM, n = 6 for 0- and 15-min time points, n = 2 for 180-min time point.

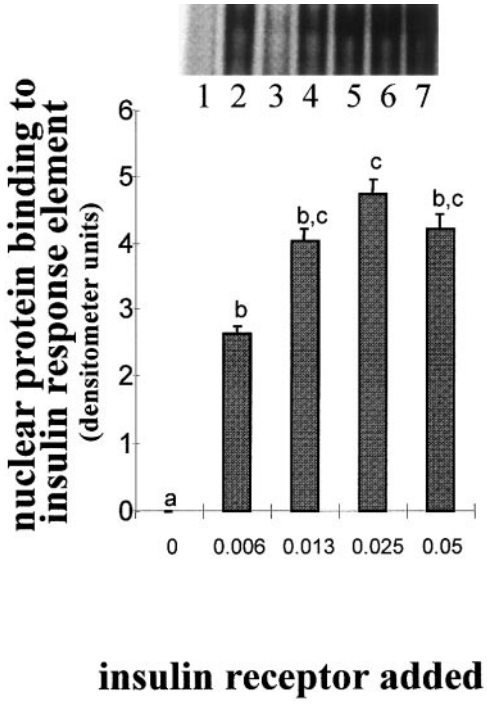

FIGURE 4 Dose-response effect of insulin receptor (IR) incubation on mouse hepatocyte nuclear protein binding to malic enzyme-insulin response element (ME-IRE). Nuclei were isolated from hepatocytes taken from mice that were food deprived for 24 h. Activated IR at varying concentrations was incubated for 15 min with nuclei extracts before the addition of the ^{32}P-labeled ME-IRE probe. Transcription factor binding was assessed by gel shift assay. Lane 1 = free probe, no nuclei added. Lanes 2, 4, 5, 6 and 7 = 0, 0.063, 0.013, 0.025 and 0.05 g/L IR incubated with nuclei, respectively. Lane 3 = excess unlabeled ME-IRE competitor added. Bands were quantitated by densitometry, and values are expressed as a change from basal in densitometer units (0 = 4.50 ± 0.13). The effect of dose of IR in ME-IRE was assessed by ANOVA. Values not sharing a common letter are significantly different ($P < 0.05$). Data represents means ± SEM, $n = 5$.

expression of two insulin responsive genes, malic enzyme and GAPDH, occurred. These results suggest that nuclear IR may play a role in transmission of the insulin signal to the hepatocyte nucleus.

IR levels in the nucleus increased rapidly after in vivo insulin stimulation, within 15 min of oral glucose administration. Similar observations have been made in insulin-stimulated adipocytes in culture (Kim and Kahn 1993). In two studies using cultured hepatocytes (Podlecki et al. 1987) and adipocytes (Smith and Jarett 1987), the kinetics of the IR internalization rate were slower. Peak accumulation of receptors in the nucleus occurred at 60 min (Podlecki et al. 1987), and there appeared to be a time delay of ~30 min of the nuclear translocation of receptor (Kim and Kahn 1993, Podlecki et al. 1987, Smith and Jarett 1987). The differences in estimated translocation rates may be a function of the different types of probes used to detect trafficking of the IR in these studies. In the studies of Podlecki et al. (1987) and Smith and Jarett (1987, [^{125}-I]-labeled insulin was covalently linked to the receptor by photoaffinity labeling before internalization of the hormone receptor complex. The covalent linkage of ligand to receptor may impair the trafficking of the receptor within the cell. Experiments show that covalently bound insulin causes routing of the IR complex to lysosomes and slow and inefficient recycling of the receptor back to the plasma membrane (Soler et al. 1989, Wong et al. 1988). In this study and in studies reported by Kim and Kahn (1993), immunodetection methods were applied to assess IR after cell fractionation. The rate of translocation of IR to the nucleus observed in this study is well within the range of rates reported for the nuclear translocation of other growth factor receptors. Studies of the kinetics of translocation of other cell surface–localized hormone receptors to the nucleus upon ligand stimulation have been reported. The time to appearance of the receptor in the nucleus varies between 10–20 min (Lobie et al. 1994) and 1–2 h (Fraser and Harvey 1992, Jiang and Schindler 1990).

Despite accumulating evidence, the idea that IR are translocated to the nucleus during the process of ligand-stimulated internalization is controversial (Kim and Kahn 1993, Soler et al. 1989). It is possible that receptors are detected at the nucleus via contamination from plasma membrane during the fractionation process involved in nuclear isolation. However, many investigators report nuclei preparations that are free of plasma membrane contamination (Kim and Kahn 1993, Wong et al. 1988). In this study, the recovery of plasma membrane 5′nucleotidase activity in nuclear fractions appears consistently to be <4% and is not increased by the oral glucose gavage. This minimal contamination could not account for the increase in IR observed in the nucleus after in vivo stimulation by oral glucose gavage. Using the same criteria, contamination by mitochondria was estimated to be <1%; however, there was contamination by microsomal membrane (8%) as determined by glucose-6-phosphatase. This finding is expected because the membranes of the endoplasmic reticulum are continuous with the outer nuclear membrane (Graham 1993).

The increase of the IR content of the hepatocyte nucleus after oral glucose gavage was associated with an increase in the β-subunit tyrosine phosphorylation during the same time period. An increase in nuclear IR β-subunit phosphorylation upon insulin stimulation has been reported previously (Kim and Kahn 1993, Wong et al. 1988), and this is demonstrated in this study. In one study using adipocytes in culture (Kim and Kahn 1993), 1 nmol/L insulin stimulated this process twofold by 5 min. These findings are consistent with the observations made in vivo in this study (1.5-fold within 15 min). In comparison, a fourfold increase in IR phosphorylation in plasma membrane of rat liver was observed after insulin administration to intact animals (Khan et al. 1989). In the study of Kim and Kahn (1993) and in this study, the time course of IR nuclear accumulation and the time course of increase in appearance of a tyrosine phosphorylated β-subunit in the nucleus were similar, implying that the receptor arrives in the nuclear membrane in a phosphorylated state. In association with the period of appearance of phosphorylated IR β-subunit in the hepatocyte nucleus, there was a decrease in tyrosine phosphorylation of a nuclear DNA-binding protein of ~30 kDa detected by immunoblotting with anti-PY. Dephosphorylation of a 30-kDa phosphotyrosine nuclear protein upon insulin stimulation has not been reported. Insulin stimulates the phosphorylation (Csermely and Kahn 1992, Csermely et al. 1993, Kim and Kahn 1994, Reucsh et al. 1995) and dephosphorylation (Daniel et al. 1996, Purrello et al. 1983) of several nuclear proteins and transcription factors. Insulin-induced changes in phosphorylation of these proteins occurs on serine and threonine residues (Csermely and Kahn 1992, Csermely et al. 1993, Daniel et al. 1996, Kim and Kahn 1994, Reucsh et al. 1995), suggesting that these phosphoproteins are not direct substrates of the IR tyrosine kinase. Insulin induces the tyrosine dephosphorylation of a nuclear protein (82 kDa) that is related to c-fos (Daniel et al. 1996); however, such a

protein was not detected in this study. With the exception of this study and that of Purrello et al. (1983), insulin regulation of protein phosphorylation was demonstrated upon stimulation of the intact cell with insulin (Csermely and Kahn 1992, Csermely et al. 1993, Daniel et al. 1996, Kim and Kahn 1994, Reucsh et al. 1995). Because the changes in phosphorylation state of the 30-kDa nuclear protein reported here are novel, it is possible that insulin stimulation of cells vs. in vitro IR treatment of nuclei produces different effects on the phosphorylation state of DNA-binding proteins. This evidence suggests that the mechanisms used in insulin signal transduction to the nucleus, when derived from the cell surface, differ from signal transduction to the nucleus when derived by direct interaction of nuclear-translocated IR with nuclear proteins.

The changes in phosphotyrosine content of a nuclear protein in this study, within the time frame of the appearance of IR tyrosine kinase in the nucleus, suggests that these changes may have been catalyzed by a tyrosine phosphatase that is regulated directly by the IR on the nuclear membrane. It is unclear how the IR, which is a tyrosine kinase, is able to inhibit incorporation of ^{32}P-phosphate into the DNA-binding phosphoprotein observed in this study. The 30-kDa phosphoprotein may be the substrate of a nuclear phosphatase whose activity is increased by insulin stimulation. Insulin action on nuclear phosphatases has been reported (Daniel et al. 1996, Purrello et al. 1983). In one study (Daniel et al. 1996), glucose treatment of mouse adipocytes induced the activation of a 38-kDa nuclear phosphatase. This protein may be an endogenous substrate of the nuclear IR tyrosine kinase. Addition of insulin to highly purified liver nuclei taken from diabetic rats resulted in a 43% decrease in the phosphorylation of ^{32}P-phosphate into nuclear proteins (Purrello et al. 1983), comparable to the observations (31% decrease) of this study. Although the presence of IR in the liver nuclei was not assessed, it is possible that in that study (Purrello et al. 1983) and this one, IR in the nucleus was involved in regulating the activity of nuclear phosphatases.

In this study, an increase of ME and GAPDH mRNA, but not of FAS mRNA was observed within 15 min. The increase in gene expression observed in this study occurred in parallel with the changes associated with IR translocation to the nucleus. IR levels and phosphorylation in the nucleus were increased 15 and 180 min after the glucose meal; however, the increase in mRNA levels appeared to be transient. This finding suggests that the induction of mRNA synthesis may be correlated with the tyrosine phosphorylation state of IR in the nucleus. Measurement of the time course of insulin induction of insulin responsive genes in vivo (Iritani et al. 1995, MacDougald et al. 1995, Saladin et al. 1995) and in cultured cells (Lin et al. 1995, Messina 1990, Stanley 1992) has been reported. In most of these studies, detection of changes in gene expression began between 2 and 4 h after stimulation (Iritani et al. 1995, Lin et al. 1995, MacDougald et al. 1995, Saladin et al. 1995, Stanley 1992). In contrast, insulin was demonstrated to increase c-fos gene expression within 5 min in cultured hepatoma cells (Messina 1990). The differences in time required for induction of the insulin-responsive gene may reflect differences in mechanisms involved in insulin signaling to the nucleus. For insulin-responsive gene expression to be affected swiftly after insulin stimulation, the mechanisms involved must be rapid such as those mediated by phosphorylation/dephosphorylation cascades and not those pathways requiring de novo protein synthesis (O'Brien and Granner 1996). There may also be specific nutrient-hormonal interactions required for the insulin response. For some genes to be regulated by insulin, the presence of glucose is required (O'Brien and Granner 1996). Although the changes observed in this study in ME gene expression were modest (35% increase from basal), they were comparable to those reported in a similar study. Administration of insulin by injection caused an increase in ME gene expression of approximately twofold (Iritani and Fukuda 1995). Although malic enzyme is not the rate-limiting enzyme of lipogenic synthesis (Volpe and Vagelos 1976), this enzyme provides the cofactor NADPH, whose availability may contribute to the flux through the lipogenic pathway.

Transcription factors that bind to the insulin responsive sequence of the malic enzyme promoter have been identified previously (Garcia-Jimenez et al. 1994). Insulin stimulation via oral glucose gavage induced an increase in the binding of transcription factors to the ME-IRE compared with the binding observed under food-deprived conditions within 15 min after glucose administration. Treatment with IR of nuclear proteins from mice in a food-deprived state caused increased binding of transcription factors to probe. The effect of IR on the binding affinity of transcription factors was dose dependent and saturable, indicating that the effect was specifically mediated by the receptor and that the effect was limited by the amount of transcription factors available in the nuclear preparation. IR induced an increase in binding affinity of transcription factors and a concomitant dephosphorylation of nuclear proteins. Taken together, these findings suggest that it is an increase in phosphatase activity and a resulting dephosphorylation of transcription factors that cause an increase in binding to the IRE within the malic enzyme gene. Transcription factor dephosphorylation resulting in an increase in DNA-binding activity is used as a mechanism for regulation of gene transcription (Hunter and Karin 1992). In one study using adipocytes, an increase in binding of the transcription factor Sp1 to the acetyl Co-A carboxylase gene IRE was attributed to dephosphorylation of the transcription factor after glucose treatment (Daniel et al. 1996). The core sequence motif CGCCTC within the malic enzyme gene may be recognized by Sp1 (Kadonaga and Tjian 1986). Because Sp1 consists of two species of 95 and 105 kDa (Kadonaga et al. 1988), it is possible that it is the binding of Sp1 to the ME-IRE that produces the two shifted bands observed in the gel-shift assay.

This study used a model of in vivo stimulation of IR, via an oral glucose dose, to show that IR signaling to the nucleus involves nuclear translocation of the activated IR tyrosine kinase. This study is the first to demonstrate ligand-dependent nuclear translocation of the IR in vivo, concomitant with in vivo insulin-induced nuclear responses. Incubation of protein isolated from the hepatocyte cell nucleus with purified IR was designed to simulate, in vitro, conditions that would occur upon translocation of the receptor to the nucleus. Hepatocyte nuclei were isolated from mice that had been food deprived for 24 h. Under these conditions, serum insulin concentrations were 100-fold below the K_m of receptor phosphorylation (2 nmol/L) (Balloti et al. 1987). Therefore, it could be assumed that insulin signaling to the nucleus via intracellular mediators could not be responsible for the effects observed. Addition of [γ^{32}P]-ATP caused the labeling of DNA-binding proteins with ^{32}P-phosphate, suggesting that one or more kinases were constitutively active in the nucleus. IR rapidly attenuated phosphate incorporation into a DNA-binding protein. This finding was supported in vivo by the observation of a decrease in tyrosine phosphorylation state of a 30-kDa DNA-binding protein within 15 min of stimulation with insulin in vivo. Thus IR, in the absence of known cytosolic mediators of insulin signaling, was able to produce a similar dephophorylation of a nuclear protein as was observed after stimulation with insulin

in vivo. This evidence suggests that IR may generate insulin signaling directly in the nucleus.

The results of this study suggest that IR in the nucleus alters the phosphorylation state of DNA-binding proteins and increases binding of transcription factors to the ME-IRE, thereby affecting the rate of transcription of that gene. The implications of this study are that rapid signaling to the nucleus exists, and it may be mediated by nuclear translocation of the IR tyrosine kinase. Direct signaling to the nuclear membrane via nuclear translocation of the IR offers a novel pathway by which dietary carbohydrate intake has rapid effects on gene expression in the liver.

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