Effect of glucagon on insulin receptor substrate-1 (IRS-1) phosphorylation and association with phosphatidylinositol 3-kinase (PI 3-kinase)

Mario J.A. Saad*, Luiz G.C. Hartmann, Daniela S. de Carvalho, Cesar A.O. Galoro, Sigisfredo L. Brenelli, Carla R.O. Carvalho

Laboratory of Cellular and Molecular Biology, Department of Internal Medicine, FCM, UNICAMP, Cidade Universitária Zeferino Vaz, Campinas, SP, Brazil 13081-970

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Abstract In the present study we have examined the levels and phosphorylation state of the insulin receptor and insulin receptor substrate 1 (IRS-1) as well as the association between IRS-1 and phosphatidylinositol 3-kinase (PI 3-kinase) in the liver and muscle of rats treated with glucagon. There was a decrease in the insulin-stimulated receptor and IRS-1 phosphorylation levels which was paralleled by a reduced association between IRS-1 and PI 3-kinase in vivo in the liver and muscle of glucagon-treated rats. These observations suggest that glucagon, probably acting through cAMP, may impair insulin signaling in the three early steps in insulin action after binding.

Key words: Glucagon; Insulin receptor; Insulin receptor substrate; Insulin action; Tyrosine kinase

1. Introduction

Insulin plays a central role in the regulation of blood glucose levels and acts in a coordinated fashion to stimulate protein and lipid metabolism by modifying the activity of various enzymes and/or transport proteins. Although the exact molecular events linking the insulin receptor tyrosine kinase to its final cellular actions remain poorly understood, several of the early steps in the insulin action cascade have been defined at a molecular level. First, insulin stimulates the tyrosine phosphorylation of cytoplasmic proteins with a relative molecular mass between 165 and 185 kDa, termed pp185 [1–3]. Insulin receptor substrate 1 (IRS-1) is a component of pp185 and is a substrate of insulin, insulin-like growth factor-1, and interleukin-4 receptors [1–4]. Following its phosphorylation, IRS-1 can associate with proteins containing Src homology 2 (SH2) domains through specific tyrosyl phosphorylation sites [5,6]. This association leads to activation of the enzyme phosphatidylinositol 3-kinase (PI 3-kinase) [7,8]. Thus, the insulin receptor, IRS-1 and PI 3-kinase represent three of the earliest steps in insulin action, each of which can be demonstrated in vivo in two of the main target tissues for the metabolic actions of insulin, namely liver and muscle [8]. Recently, we demonstrated alterations in these early steps of insulin action in liver and muscle of animal models of insulin resistance [9–11]

It has long been known that an increase in intracellular cyclic AMP levels induce insulin resistance at both receptor and post-receptor levels [12–15]. Using purified receptors and artificial substrates in vitro, decreased insulin receptor phosphorylation and kinase activity have been reported as a consequence of increased cAMP and cAMP kinase [14,15], although such a change is not always observed [16]. In the present study, we have examined the phosphorylation state of the insulin receptor and IRS-1 as well as the association of the latter with PI 3-kinase in the liver and muscle of glucagon-treated rats stimulated with insulin in vivo.

2. Materials and methods

2.1. Materials

The reagents for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting were from Bio-Rad (Richmond, CA). *N*-2-Hydroxyethyl-piperarine-*N*-2 ethanesulfonic acid (HEPES), phenylmethylsulfonylfluoride (PMSF), aprotinin, dithiothreitol (DTT), Triton X-100, Tween 20, glycerol, glucagon and bovine serum albumin (BSA, fraction V) were from Sigma Chemical Co. (St. Louis, MO). Protein A-Sepharose 6MB was from Pharmacia (Uppsala, Sweden), [^{125}I]protein A was from ICN Biomedicals (Costa Mesa, CA), and nitrocellulose paper (BA85, 0.2 mm) was from Schleicher & Schuell. Sodium amobarbital (Amytal) and human recombinant insulin (Humulin R) were from Eli Lilly. Monoclonal antiphosphotyrosine antibody and anti-rat-PI 3-kinase antiserum were from UBI (Lake Placid, NY). Anti-insulin receptor antibody and anti-IRS-1 antibodies were raised in rabbits using a synthetic peptide derived from the amino acid sequence of the C-terminus of the proteins, as previously described [11], and were kindly provided by Dr. C.R. Kahn and M.F. White (Joslin Diabetes Center, Boston, MA).

2.2. Methods

2.2.1. Animals and tissue extracts. Male rats (130–180 g) were allowed access to standard rodent chow and water ad libitum. The animals were studied after 12 h of fasting. The studies were performed in parallel for the control and treated rats in each pair.

Rats were anesthetized with sodium amobarbital (15 mg/kg body weight, i.p.) and used in experiments as soon as anesthesia was assured by loss of pedal and corneal reflexes. Initially rats were injected i.p. with glucagon 100 μg/100 g rat or an equal volume of saline (control group). Five minutes later, the abdominal cavity was opened, the portal vein exposed, and 0.5 ml of normal saline (0.9% NaCl) with or without 10^{-5} M insulin was injected. At 30 s, the livers were removed, minced coarsely and homogenized immediately in approximately 10 volumes of solubilization buffer A in a water bath maintained at 100°C with a Polytron PTA 20S generator (Brinkmann Instruments model PT 10/35) operated at maximum speed (setting 10) for 30 s. The solubilization buffer A was composed of 1% SDS, 50 mM HEPES (pH 7.4), 100 mM sodium pyrophosphate, 100 mM sodium fluoride, 10 mM EDTA, and 10 mM sodium vanadate. The homogenate was boiled for 10 min and then cooled in an ice bath for 40 min.

Approximately 90 s after insulin injection, hindlimb muscles were excised and then homogenized with a Polytron in 6 volumes of homogenization buffer A at 100°C. The extracts were centrifuged at 15,000 rpm at 4°C in a Beckman 70.1 Ti rotor for 60 min to remove insoluble material, and the supernatant was used as a sample. In some experiments, the tissues were extracted at 4°C with homogenization buffer B (same as buffer A except that 1% Triton X-100 replaced 1% SDS and 2 mM PMSF and 0.1 mg aprotinin/ml were added), and after centrifu-

*Corresponding author. Fax: (55) (19) 2393114.

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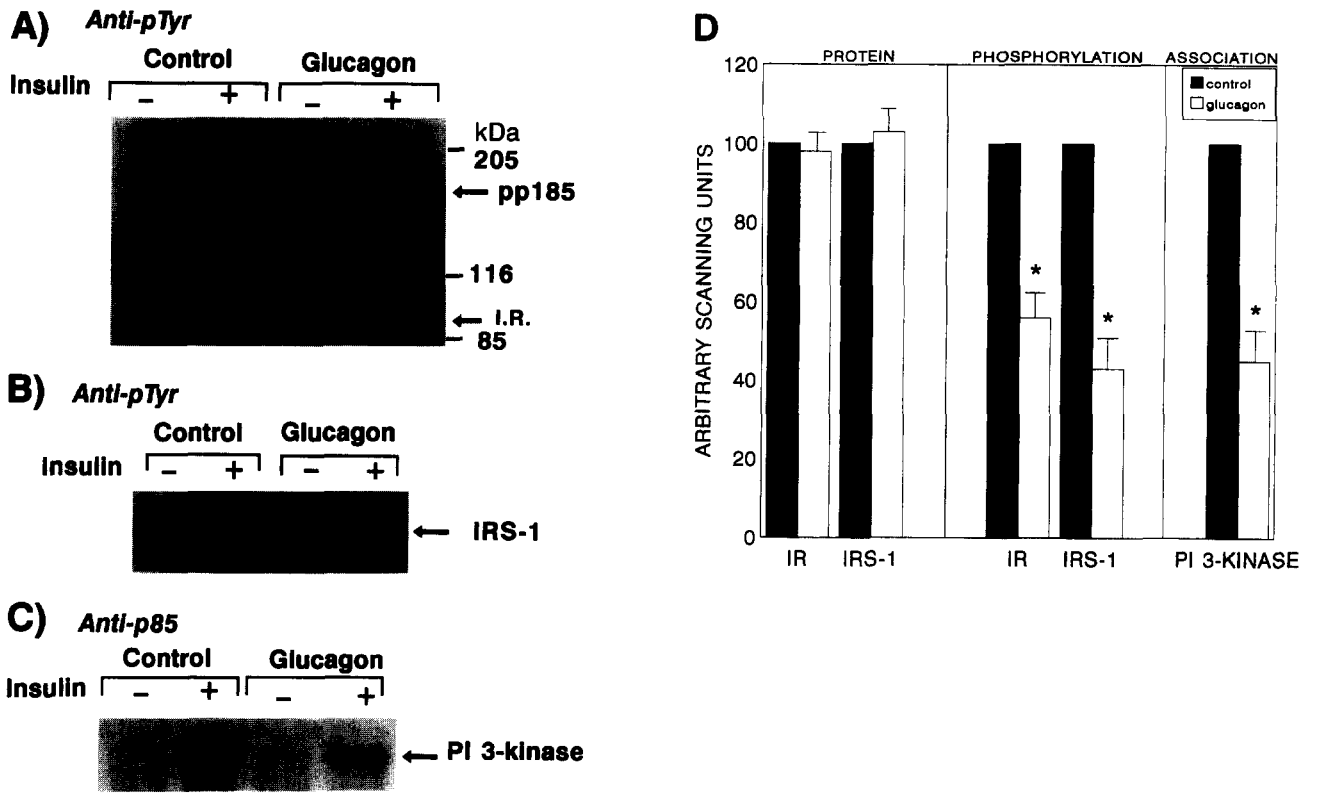

Fig. 1. Insulin-stimulated tyrosine phosphorylation in the intact liver of glucagon-treated rats. Rats were anesthetized, and the abdominal wall incised to expose the viscera. Normal saline (lanes 1,3) or 10^{-5} M insulin (lanes 2,4) was infused into the portal vein as a bolus injection and 30 s later the livers were excised and homogenized in extraction buffer A at 100°C as described in section 2.2. After centrifugation, aliquots containing the same amounts of protein were resolved on 6% SDS-polyacrylamide gels, transferred to nitrocellulose filters, and detected with anti-phosphotyrosine antibody (panel A), anti-IRS-1 antibody , or anti-insulin receptor antibody, and [^{125}I]protein A and subjected to autoradiography. Panels B and C show the immunoblotting of tyrosyl phosphorylated proteins and PI 3-kinase in anti-IRS-1 immunoprecipitates from the liver of control and glucagon-treated rats. The liver proteins were extracted and processed in extraction buffer B as described in section 2, and then incubated at 4°C with anti-IRS antibody and Protein A-Sepharose 6MB. Immunoprecipitated proteins were analyzed by immunoblotting with anti-phosphotyrosine (panel B) and anti-PI 3-kinase (85 kDa subunit) (panel C) antibodies and [^{125}I]protein A and subjected to autoradiography. The data shown are representative of several independent experiments. (D) Insulin receptor and IRS-1 protein levels and tyrosine phosphorylation in the liver of control and glucagon-treated rats. Scanning densitometry of autoradiograms was performed on 8 experiments to determine the insulin receptor and IRS-1 concentration, 9 experiments for the tyrosine phosphorylation levels of both proteins and 6 experiments for PI 3-kinase associated with IRS-1. The data are expressed as the mean ± S.E.M. and are normalized per protein. *, significant differences at least at $P < 0.05$.

gation the supernatant was used for immunoprecipitation with anti-IRS-1 antibody.

2.2.2. Protein analysis by immunoblotting. The samples were treated with Laemmli sample buffer [17] with 100 mM DTT and heated in a boiling water bath for 4 min. For total extracts, similar size aliquots of sample (150 μg of protein) were subjected to SDS-PAGE (6% Tris acrylamide) in a Bio-Rad miniature slab gel apparatus. Electrotransfer of proteins from the gel to the nitrocellulose membrane was performed for 2 h at 120 V (constant) in the Bio-Rad miniature transfer apparatus (Mini-Protean) as described by Towbin et al. [18] but with 0.02% SDS added to the transfer buffer to enhance the elution of high molecular mass proteins. Non-specific protein binding to the nitrocellulose was reduced by preincubating the filter overnight at 4°C in blocking buffer (3% BSA, 10 mM Tris, 150 mM NaCl, and 0.02% Tween 20). The prestained molecular weight standards used included myosin (205 kDa), β galactosidase (116 kDa), bovine serum albumin (80 kDa), and ovalbumin (49.5 kDa).

The nitrocellulose blot was incubated with anti-phosphotyrosine antibodies, anti-insulin receptor antibodies, anti-IRS-1 antibodies or anti-PI 3-kinase antibodies diluted in blocking buffer for 4 h at 22°C and washed for 60 min with the blocking buffer without BSA. The blots were then incubated with 2 mCi of [^{125}I]protein A (30 mCi/mg) in 10 ml of blocking buffer for 1 h at 22°C and washed again as described above for 2 h. ^{125}I-Protein A bound to the antibodies was detected by autoradiography using preflashed Kodak XAR film with Cronex Lightning Plus intensifying screens at −70°C for 12–48 h. Band intensities were quantitated by optical densitometry (Molecular Dynamics) of the developed autoradiogram.

2.3. Other

Protein determination was performed by the Bradford dye method [19] using the Bio-Rad reagent and BSA as the standard.

2.4. Statistics

Experiments were always performed using samples from glucagon-treated animals in parallel with a control group. Comparisons were made using student's paired and unpaired t test as appropriate. The level of significance employed was $P < 0.05$.

3. Results and discussion

In the present study, we have evaluated the effect of glucagon on insulin receptor and IRS-1 phosphorylation and on the association of PI 3-kinase with IRS-1 in the liver and muscle of rats treated with glucagon. Acute glucagon-treatment had no effect on the insulin receptor and IRS-1 protein levels in liver

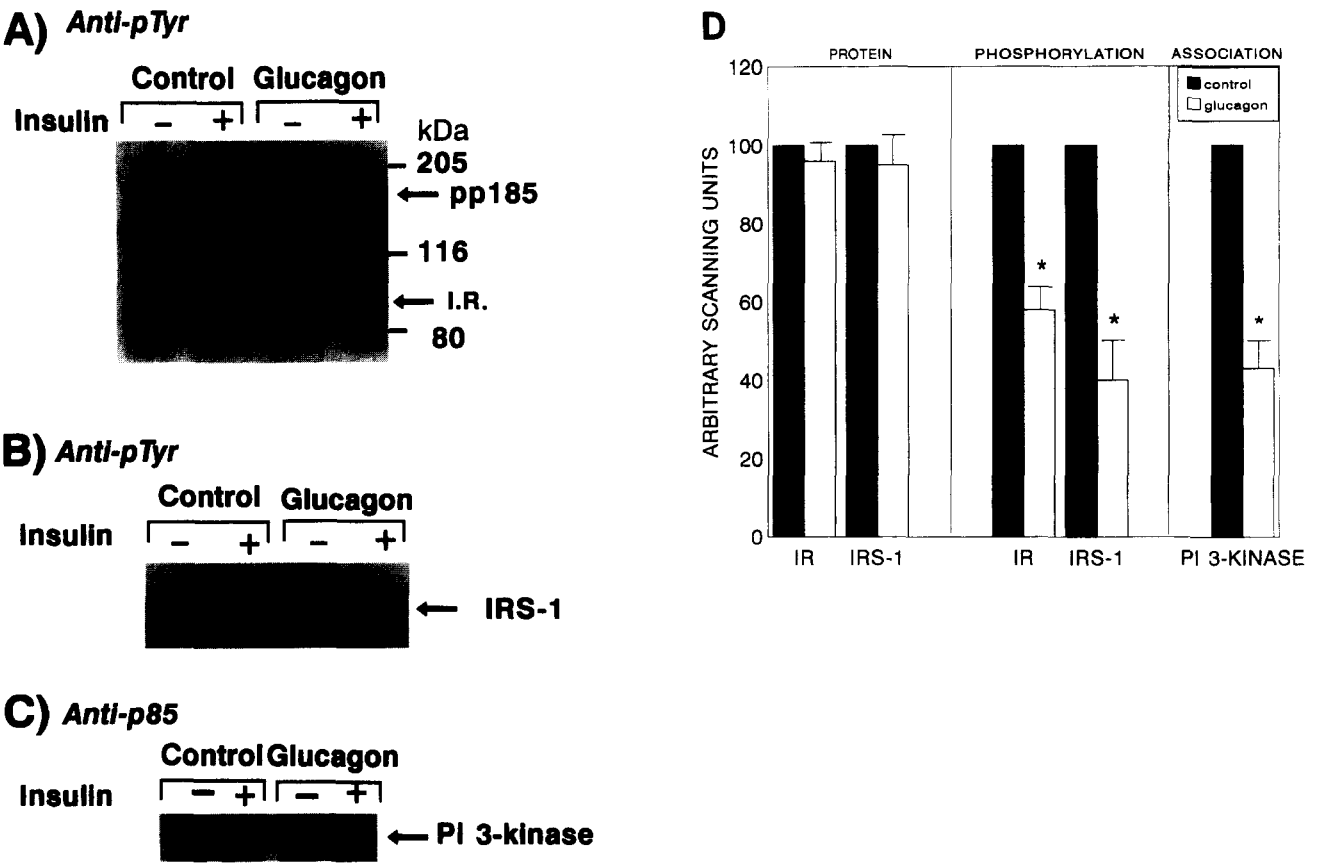

Fig. 2. Insulin-stimulated tyrosine phosphorylation in the intact muscle of rats treated with glucagon. Rats were anesthetized, the abdominal wall incised to expose viscera. Normal saline (lanes 1,3) or 10^{-5}M insulin (lanes 2,4) was infused into the portal vein as a bolus injection and 90 seconds later the muscle was excised and homogenized in extraction buffer A at 100°C for 5 min as described in section 2.2. After centrifugation aliquots containing the same amount of protein were resolved on 6% SDS-polyacrylamide gels, transferred to nitrocellulose, and detected with anti-phosphotyrosine antibody (panel A), anti-IRS-1 antibody or anti-insulin receptor antibody and [^{125}I]protein A and then subjected to autoradiography. Panels B and C show the immunoblot of tyrosyl phosphorylated proteins and PI 3-kinase in anti-IRS-1 immunoprecipitates from the muscle of control and glucagon-treated rats. The muscle proteins were extracted and processed in extraction buffer B as described in the Methods, solubilized and incubated at 4°C with anti-IRS antibody and Protein A-Sepharose 6MB. The immunoprecipitated proteins were analyzed by immunoblotting with anti-phosphotyrosine (panel B) and anti-PI 3-kinase (85 kDa subunit) (panel C) antibodies and [^{125}I]protein A and then subjected to autoradiography. The data shown are representative of several independent experiments. (D) Insulin receptor and IRS-1 protein levels and tyrosine phosphorylation in muscle of glucagon-treated rats. Scanning densitometry was performed on autoradiograms from 8 experiments with insulin receptor concentration, 12 experiments with IRS-1 protein levels, 8 experiments for the tyrosine phosphorylation of both proteins and 6 experiments for PI 3-kinase associated with IRS-1. The data are expressed as the mean ± S.E.M. and are normalized per protein. *, differences significant at least at $P < 0.05$.

and muscle as determined by immunoblotting with an antibody to the C-terminus of the insulin receptor and an antibody to the C-terminus of IRS-1, respectively (Figs. 1D and 2D).

Following insulin infusion into the portal vein, a phosphotyrosine band of 95 kDa, previously identified as the insulin receptor β-subunit, appeared and became prominently phosphorylated. The level of phosphorylation of this band was reduced to 56 ± 7% ($P < 0.01$) in the liver (Fig. 1A,D) and to 58 ± 7% ($P < 0.01$) in the muscle (Fig. 2A,D) of rats treated with glucagon. This is consistent with previous studies which demonstrated that an increase in the cAMP content of cells alters the phosphorylation state and protein kinase activity of the insulin receptor [14,15], although such a response is not always observed [16].

In anti-phosphotyrosine blots of whole tissue extracts, in addition to the 95 kDa band seen after insulin injection, a broad protein band migrating between 165 and 185 kDa was also detectable (Figs. 1A and 2A). This band is known as pp185 and IRS-1 is one component of this band [20,21]. The phosphorylation of pp185 (kinase activity of insulin receptor toward endogenous substrates) was reduced by 51 ± 12% ($P < 0.01$) in the liver (Fig. 1A) and by 62 ± 11% ($P < 0.01$) in the muscle (Fig. 2A) of glucagon-treated rats. In order to characterize IRS-1 phosphorylation after insulin stimulation, we immunoprecipitated liver and muscle extracts with anti-IRS-1 antibody and immunoblotted these with anti-phosphotyrosine antibody (Figs. 1B and 2B). The results showed that after insulin stimulation, compared to the controls IRS-1 phosphorylation decreased by 57 ± 11% ($P < 0.01$) and 60 ± 9% ($P < 0.01$) in the liver and muscle, respectively, of animals treated with glucagon (Figs. 1B,D and 2B,D).

Previous studies [7,8,22–25] have suggested that there is a relatively stable, high affinity interaction between IRS-1 and the 85 kDa subunit of PI 3-kinase, such that both proteins are co-precipitated by antibodies to either protein. In samples from muscle and liver previously immunoprecipitated with anti-IRS-1 antibody and subsequently incubated with antibodies directed against the 85 kDa subunit of PI 3-kinase, there was little

detectable PI 3-kinase immunoreactivity in the basal state in either the normal or glucagon-treated animals (Figs. 1C and 2C). After stimulation with insulin, a band with the expected molecular weight of the regulatory subunit of PI 3-kinase (85 kDa) was present in anti-IRS-1 antibody immunoprecipitates from the muscle (Fig. 2C) and liver (Fig. 1C) of both groups of rats, which was consistent with the formation of a stable association between IRS-1 and PI 3-kinase. However, the amount of PI 3-kinase associated with IRS-1 was reduced by $55 \pm 9\%$ ($P < 0.01$) in liver and by $57 \pm 8\%$ ($P < 0.01$) in the muscle of glucagon-treated rats, demonstrating less association between IRS-1 and PI 3-kinase. Recent evidence from various sources suggests that the IRS-1/PI 3-kinase pathway may be linked to the activation of glucose transport [26–28]. Interestingly, Sato et al. [29] demonstrated that glucagon inhibits activation of glucose transport by insulin in rat adipocytes, mainly through a postbinding process. Based on these results, it seems reasonable to speculate that a reduction in the interaction and activity of the IRS-1/PI 3-kinase pathway may play a role in the reduced glucose uptake induced by glucagon in peripheral tissues [29].

The mechanism(s) by which glucagon alters the three early steps in insulin action are not completely underestood but at least two possibilities should be considered. First, it is well known that agents which raise intracellular cAMP levels increase phosphorylation of the insulin receptor at serine and threonine residues, reduce insulin-mediated receptor phosphorylation on tyrosine, and inhibit the insulin-dependent tyrosine protein kinase activity of the receptor. Thus, cAMP may attenuate insulin action by altering the state of phosphorylation of the insulin receptor [14–16]. It is not known wether an increase in intracellular cAMP also induces a serine phosphorylation in IRS-1. However, since insulin receptor kinase activity is reduced, a reduction in IRS-1 phosphorylation and hence in the association between IRS-1/PI 3-kinase is also expected. Another possibility arises from recent data showing that an increase in cellular cAMP through activating protein kinase A (PKA) increases the activity of endogenous phosphotyrosine phosphatase (PTPase), thus leading to a sequence of dephosphorylation [30].

Our results may have importance in the crosstalk between two major signal pathways. Recently, it was demonstrated that hormones, such as glucagon which increase cyclic AMP concentration inhibit the transmission of growth stimulatory signals which depend on receptors with tyrosine kinase activity and the MAP kinase pathway [31,32], probably by increasing Raf-1 phosphorylation at serine-43 in the regulatory domain. In the transmission of the insulin signal through its tyrosine kinase receptor, the Raf-1 and MAP kinase pathways are downstream to IRS-1. The present study has demonstrated that glucagon, probably acting through cyclic AMP and PKA, inhibits insulin signal transmission at the three early steps, i.e. insulin receptor and IRS-1 phosphorylation and the interaction between IRS-1 and PI 3-kinase. Thus, at least for insulin signal transmission, the inhibition induced by hormones that increase cyclic AMP levels may also be upstream to Raf-1.

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References

[1] White, M.F., Maron, R. and Kahn, C.R. (1985) Nature 318, 183–186.
[2] Rothenberg, P.L., Lane, W.S., Karasik, A., Backer, J., White, M.F. and Kahn, C.R. (1991) J. Biol. Chem. 266, 8302–8311.
[3] Sun X.J., Rothenberg, P.L., Kahn, C.R., Backer, J.M., Araki, E., Wilden, P., Cahill, D.A., Goldstein, B.J. and White, M.F. (1991) Nature 352, 73–77.
[4] Wang, L., Keegan, A.D., Li, W., Lienhard, G.E., Pacini, S., Gutkind, W.E., Myers, M.G., Sun, X.J., White, M.F., Aaronson, S.A., Paul, W.E. and Pierce, J.H. (1993) Proc. Natl. Acad Sci USA 90, 4032–4036.
[5] Kuhne, M.R., Pawson, T., Lienhard, G.E. and Feng, G.H. (1993) J. Biol. Chem. 268, 11479–11481.
[6] Skolnik, E.Y., Lee, C.H., Batzer, A., Vicentini, L.M., Zhou, M., Myers, M.G., Backer, J.M., Ulrich, A., White, M.F. and Schlessinger, J. (1993) EMBO J. 12, 1926–1936.
[7] Backer, J.M., Myers Jr., M.G., Shoelson, S.E., Chin, D.J., Sun, X.J., Miralpeix, M., Hu, P., Margolis, B., Skolnik, E.Y., Schlessinger, J. and White, M.F. (1992) EMBO J. 11, 3469–3479.
[8] Folli, F., Saad, M.J.A., Backer, J.M. and Kahn, C.R. (1992) J. Biol. Chem. 267, 22171–22177.
[9] Saad, M.J.A., Araki, E., Miralpeix, M., Rothenberg, P.L., White, M.F. and Kahn, C.R. (1992) J. Clin. Invest. 90, 1839–1849.
[10] Saad, M.J.A., Folli, F., Kahn, J.A. and Kahn, C.R. (1993) J. Clin. Invest. 92, 2065–2072.
[11] Folli, F., Saad M.J.A., Backer, J.M. and Kahn, C.R. (1993) J. Clin. Invest. 92, 1787–17994.
[12] Kirsch, D., Kemmler, W. and Haring, H.U. (1983) Biochem. Biophys. Res. Commun. 115, 389–405.
[13] Pessin, J.E., Gitomer, W., Oka, Y., Oppenheimer, C.L. and Czech, M.P. (1983) J. Biol. Chem. 258, 7386–7394.
[14] Stadmauer, L. and Rosen, O.M. (1986) J. Biol. Chem. 261, 3402–3407.
[15] Roth, R.A. and Beaudoin, J. (1987) Diabetes 36, 123–126.
[16] Issad, T., Young, S.W., Tavaré, J.M. and Denton, R.M. (1992) FEBS Lett. 296, 41–45.
[17] Laemmli, U.K. (1970) Nature 227, 680–685.
[18] Towbin, H., Staehlin, J. and Gordon, J. (1979) Proc. Natl. Acad. Sci. USA 76, 4350–4354.
[19] Bradford, M.M. (1976) Anal. Biochem. 72, 248–254.
[20] Tamemoto, H., Kadowaki, T., Tobe, K., Yagi, T., Sakura, H., Hayakawa, T., Terauchi, Y., Ueki, K., Kaburagi, Y., Satoh, S., Sekihara, H., Yoshioka, S., Harikoshi, H., Furuta, Y., Ikawa, Y., Kasuga, M., Yazaki, Y. and Aizawa, S. (1994) Nature 372, 182–186.
[21] Araki, E., Lipes, M.A., Patti, M.E., Bruining, J.C., Haag III, B., Johnson, R.S. and Kahn, C.R. (1994) Nature 372, 186–190.
[22] Lamphere, L., Carpenter, C.L., Sheng, Z.F., Kallen, R.G. and Lienhard, G.E. (1994) Am. J. Physiol 266, E486–E494.
[23] Giorgetti, S., Balloti, R., Kowalski-Chauvel, A., Tartare, S. and VanObberghen, E. (1993) J. Biol. Chem. 268, 7358–7364.
[24] Kelly, K.L. and Ruderman, N.B. (1993) J. Biol. Chem. 268, 4391–4398.
[25] Yonezawa, K.H., Ueda, H., Hara, K., Nishida, K., Ando, A., Chavanieu, A., Matsuba, H., Shii, K., Yokono, K., Fukui, Y., Calas, B., Grigorescu, F., Dhand, R., Gout, I., Otsu, M., Waterfield, M.D. and Kasuga, M. (1993) J. Biol. Chem. 267, 25958–25966.
[26] Kanai, F., Ito, K., Todaka, M., Hayashi, H., Kamahara, S., Ishii, K., Okada, T., Hazeki, O., Ui, M. and Ebina, Y., (1993) Biochem. Biophys. Res. Commun. 196, 762–768.
[27] Cheatham, B., Vlahos, C.J., Cheatham, L., Wang, L., Blenis, J. and Kahn, C.R. (1994) Mol. Cell. Biol. 14, 4902–4911.
[28] Quon, J.M., Butte, A.J., Zarnowski, M.J., Sesti, G., Cushman, S.W. and Taylor, S.I. (1994) J. Biol. Chem. 269, 27920–27924.
[29] Sato, N., Irie, M., Kajinuma, H., Suzuki, K. (1990) Endocrinology 127, 1072–1077.
[30] Wilson, G.F. and Kaczmarek, L.K. (1993) Nature 366, 433–438.
[31] Cook, S.J. and MacCornick, F. (1993) Science 262, 1069–1072.
[32] Marx, J. (1993) Science 262, 988–990.