Potentiation of Insulin Stimulation of Phosphatidylinositol 3-Kinase by Thiazolidinedione-derived Antidiabetic Agents in Chinese Hamster Ovary Cells Expressing Human Insulin Receptors and L6 Myotubes

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Thiazolidinedione derivatives are insulin-sensitizing agents with proven antidiabetic activities in vivo. To explore the mechanism of action of this class of compounds, the effects of pioglitazone, CP-86,325, and AD-5075 on elements of the insulin signal transduction pathways were studied in Chinese hamster ovary cells overexpressing human insulin receptor (CHO-T) and L6 myotubes. In CHO-T cells, the binding of insulin to its receptor and the insulin-stimulated tyrosine kinase activity of the receptor were not altered by pioglitazone or CP-86,325. In contrast, treatment of CHO-T cells with the compounds resulted in significant increases in insulin-stimulated phosphatidylinositol (PI) 3-kinase activity. This insulin-enhancing effect was also observed in L6 myotubes treated with CP-86,325. The augmentations in kinase activity observed in CHO-T cells correlated with increases in the amount of PI 3-kinase (p85 subunit) in anti-phosphotyrosine immunoprecipitates of cell lysates. No gross changes in the tyrosine phosphorylation state of the insulin receptor substrate-1 were detected in insulin-stimulated CHO-T cells following treatment with the compounds. Furthermore, the compounds did not enhance insulin stimulation of mitogen-activated protein kinase or DNA synthesis in CHO-T cells. Thus, thiazolidinedione-derived antidiabetic agents may act as insulin sensizers by augmenting insulin stimulation of PI 3-kinase activity in a rather specific manner.

Insulin elicits a diverse array of biological responses by binding to its specific receptor (1). The insulin receptor (IR)1 is a heterotetrameric protein consisting of two extracellular α subunits and two transmembrane β subunits. The binding of the ligand to the α subunit of IR not only concentrates insulin at its site of action but also induces conformational changes in the receptor, which in turn stimulate the tyrosine kinase activity intrinsic to the β subunit of the IR. Extensive studies have indicated that the ability of the receptor to autophosphorylate and phosphorylate intracellular substrates is essential for its mediation of the complex cellular responses of insulin (2-7). One of the immediate substrates of the insulin receptor tyrosine kinase is IRS-1, a cytoplasmic protein with an apparent molecular mass of approximately 185 kDa (8). IRS-1 contains numerous YXXM tyrosine phosphorylation motifs that interact with downstream effectors of insulin containing src homology (SH) 2 domains (8). One such SH2-containing protein is phosphatidylinositol (PI) 3-kinase, an enzyme composed of a regulatory subunit (p85) and a catalytic subunit (p110) that phosphorylates the D3 position of the inositol ring of PI and its phosphorylated derivatives (9). The binding of p85 to IRS-1 through interactions between the SH2 domain of the former and tyrosine-phosphorylated YXXM motifs of the latter activates the enzyme (10-18). This activation process is implicated in insulin-signalling transduction within cultured cells and in vivo (14-18).

Insulin is essential for maintaining glucose homeostasis and regulating carbohydrate, lipid, and protein metabolism (1). Decreased cellular response to insulin or perturbation of the insulin-signaling pathways are associated with a number of pathological states. Indeed, marked insulin resistance is found in patients with non-insulin-dependent diabetes mellitus (NIDDM) (19). Thiazolidinediones are derivatives of a class of antidiabetic agents with in vivo insulin-sensitizing activities (20). These agents lower plasma glucose and triglyceride levels in animal models of obesity and NIDDM (21-23). Furthermore, treatment of insulin-resistant animals with the compounds resulted in enhancement of insulin activity in vitro and in vivo in the three major target tissues of insulin action. This included stimulation of glucose uptake, glucose oxidation, and lipogenesis in adipose and muscle as well as stimulation of lipogenesis and inhibition of glucose output by the liver (24-26). Pilot clinical trials with one thiazolidinedione-derived compound, CS-045, in patients with NIDDM have demonstrated the efficacy of this class of compounds as oral antidiabetic agents (27, 28). Despite more than a decade of research, however, little is known about the molecular mechanism of action of these insulin sensitizers.

The present study was initiated to investigate the effects of these antidiabetic agents on various steps in the insulin signal transduction pathways. The results indicate that the thiazolidinediones derivatives may augment cellular insulin sensitivity by potentiating insulin stimulation of PI 3-kinase activity. This potentiation correlates positively with the amount of the PI 3-kinase associated with tyrosine-phosphorylated proteins. Presumably, these compounds augment the association of p85 with tyrosine-phosphorylated IRS-1.

EXPERIMENTAL PROCEDURES

Materials—The anti-phosphotyrosine monoclonal antibody PY20 was purchased from Transduction Laboratories. Polyclonal antibodies

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1 The abbreviations used are: IR, insulin receptor; CHO, Chinese hamster ovary; CHO-T, CHG cells expressing human insulin receptor; IRS-1, insulin receptor substrate-1; MAP, mitogen-activated protein; PAGE, polyacrylamide gel electrophoresis; PI, phosphatidylinositol; PIO, pioglitazone; CS-045, (5-[4-(5-ethyl-2-pyridyl)ethoxy]benzyl)-2,4-thiazolidinedione; CP, CP-86,325 (5-[4-[3-(5-methyl-2-phenyl-4-oxazolyl)-2-hydroxyethoxy]benzyl]-3,4-thiazolidinedione; SH, src homology; NIDDM, non-insulin-dependent diabetes mellitus.
against the p85 subunit of the PI 3-kinase were obtained from UBI. Radiolabeled compounds were purchased from DuPont NEN. Phloretazine (5-[4-[(2-ethyl-hydroxymethyl)ethoxy]benzyl]-2,4-thiazolidinedione) (PIO), CP-86,325 (5-[4-[(3-5-methyl-2-phenyl-4-oxazolyl)propionyl]benzyl]-2,4-thiazolidinedione (CP)), and AD-5075 (5-[4-[(2-ethyl-hydroxymethyl)2-phenyl-4-oxazolyl]2-hydroxyethylbenzyl]-2,4-thiazolidinedione (AD)) were kindly provided by J. Berger, Robert Marquis, Dong Ok, Conrad Santini, and Lihu Yang of Merck Research Laboratories. Other chemicals were from Sigma.

**Antibody Production**—Polyclonal antibodies against IRS-1 I were produced in rabbits using a 20-amino acid peptide as an antigen. The sequence of this peptide corresponds to a 20-amino acid sequence 6–25 of amino acid sequence 6 of IRS-1 (8). The antisera were affinity-purified on an antigen-linked Affi- Gel (Bio-Rad) column according to published procedures (29).

**Cell Culture and Treatment**—Chinese hamster ovary cells expressing human insulin receptor (CHO-T) were a gift from Dr. Richard Roth, Stanford University, CA. CHO or CHO.T cells were treated with the compounds for 24 h and subsequently stimulated with 10% fetal bovine serum (Hyclone), 100 units/ml penicillin, and 100 μg/ml streptomycin (Life Technologies, Inc.) at 37°C in 5% CO2. L6 cells (kindly provided by Dr. Amira Klip, The Hospital for Sick Children, Toronto, Canada) were cultured in a α-minimal essential medium (Life Technologies, Inc.) containing 2% fetal bovine serum (Life Technologies, Inc.), 100 units/ml penicillin G and 100 μg/ml streptomycin and sulfoxide at 37°C in a humidified atmosphere of 5.5% CO2. Cells were allowed to grow and fuse into myotubes as described previously (30). For experiments, the cells were treated in the appropriate serum-free media containing thiazolidinedione derivatives dissolved in dimethyl sulfoxide. Cells were washed once with phosphate-buffered saline and one with buffer E containing 60 μg β-glycophorin, pH 7.3, 1 mg benzamidine, 1.5 mg EGTA, 0.1 mg sodium orthovanadate, 1 mg dithiothreitol, and 10 μg/ml each of leupeptin and aprotinin and stored into buffer E (0.3 ml/100-mm plate). The lysates were homogenized by 10 passages through 24-gauge needles and centrifuged at 100,000 × g for 15 min. The kinase assay was initiated by mixing 12.5 μl of cleared lysates with 12.5 μl of reaction mixture containing 0.66 mg/ml myelin basic protein, 2 μl dithiothreitol, 2 μl sodium orthovanadate, 20 μl MgCl2, 5 μl EGTA, 0.2 μl [γ-32P]ATP (0.2 μCi/μl), 4 μg rabbit protein kinase A inhibitor, and 20 μg calmodulin. After incubation at 25°C for 5 min, the reaction was terminated by spotting 20 μl of the mixture on a P81 (Whatman) phosphocellulose filter and immersing the filter in 1% H3PO4. The filters were rinsed four times with 1% H3PO4 and once with 95% ethanol. The radioactivity bound to the filters was determined by Cerenkov counting.

**MAP Kinase Assay**—The assay was performed essentially as described previously (31). CHO-T cells were plated in 24-well dishes and cultured to 80% confluence. The cells were treated with thiazolidinedione derivatives for 24 h and then washed twice with ice-cold buffer A (Hepes-buffered saline, pH 7.4, 0.3 mM CaCl2). Next, the cells were incubated at 37°C for 5 min with 50 μl of buffer B (100 μM Hepes pH 7.8, 120 mM NaCl, 1.2 mM MgSO4, 1 mM EDTA, 150 mM sodium acetate, 10 mM glucose, 1% bovine serum albumin) containing various concentrations of unlabeled insulin and [32P]insulin (40,000 cpm/well). The cells were then washed twice with buffer A and lysed with 0.5 ml of 0.5% Triton X-100. The radioactivity in the lysates was quantitated in a γ counter. Nonspecific binding was assessed in the presence of 1 μM unlabeled insulin.

**Tyrosine Kinase Assay**—The IR tyrosine kinase activity was determined using a previously described procedure (31). Briefly, confluent CHO or CHO.T cells were treated with the compounds for 5 h followed by incubation with 100 nM insulin for 15 min at 37°C. The cells were lysed in buffer C containing 50 mM Hepes pH 7.6, 5 mM EDTA, 5 mM EGTA, 1% Triton X-100, 150 mM NaCl, 1 mM sodium orthovanadate, 20 mM sodium fluoride, 20 mM sodium pyrophosphate, 1 mM phenylmethylsulfonyl fluoride, and 1 mg/ml bacitracin. Forty microliters of lysate were added to 95-well microplates as described previously (8). The lysates were coated with a monoclonal antibody against the IR (Oncogene Science). After incubation for 16 h at 4°C, the wells were washed three times with WGBT (50 mM Hepes pH 7.6, 150 mM NaCl, 0.1% Triton, 0.1% Tween 20, 0.1% bovine serum albumin). Twenty microliters of the kinase reaction mixture (150 mM Hepes pH 7.6, 150 mM NaCl, 5 mM MgCl2, 5 mM MnCl2, 0.1% Triton X-100, 1 mg/ml poly(Glu:Tyr) (4:1), 2 μC of carrier-free [γ-32P]ATP) were added to one set of wells, and the incubation was continued at 25°C for 30 min. The reaction was terminated by spotting 10 μl of the reaction mixture on Whatman No. 3MM filter strips. The filter strips were treated with trichloroacetic acid, and radioactivity was detected by Cerenkov counting. The amount of IR captured in the wells was determined by [32P]insulin binding to the second set of wells. Forty microliters of [35S] insulin (1000 cpm/μl) in WGB ask were added to each well, and the incubation was continued at 4°C for 4 h. The wells were then washed and counted. The tyrosine kinase activities were normalized against the amount of [32P]insulin specifically bound to captured IR.

**PI 3 Kinase Assay**—CHO, CHO-T, and L6 cells were pretreated with the compounds for 5 h and subsequently stimulated with 100 nM insulin for 5 min at 37°C. Lysates were prepared, and solubilized proteins were subjected to immunoprecipitation with monoclonal anti-IR antibodies conjugated to agarose beads (Sigma). The beads were washed, and PI 3-kinase activity was determined directly in the immunoprecipitates as described previously (15). The reaction was carried out at 25°C for 10 min in a mixture (50 μl) containing 0.2 mg/ml PI, 20 μM dithiothreitol, 0.6 mM MgCl2, 0.2 mM [γ-32P]ATP (40 μM and 0.1 μCi/μl) and terminated by the addition of 15 μl of 4 N HCl and 130 μl of chloroform/methanol (1:1).
amounts of the $^{125}$I-insulin bound to wells containing IR from control or compound-treated cells varied by less than 10%. As expected, insulin caused a dose-dependent stimulation of IR tyrosine kinase activity in CHO-T cells, and the maximum stimulation was 26-fold compared with unstimulated cells. Preincubation of CHO-T cells with CP or PI0 did not alter the sensitivity or maximal responsiveness of their IR tyrosine kinase activity to insulin (Fig. 2A). The inability of the compounds to affect IR autophosphorylation was demonstrated by anti-phosphotyrosine immunoblot analysis of CHO-T cell proteins (Fig. 2B). Pretreatment of insulin-stimulated cells with thiazolidinedione derivatives caused no further detectable increase in the tyrosine phosphorylation level of the 97 kDa band corresponding to the receptor β subunit.

**PI 3-Kinase Activation—**CHO-T cells were preincubated alone or in the presence of PI0 or CP for 5 h. They were then stimulated with increasing concentrations of insulin for 5 min at 37 °C. The cells were solubilized, and cellular proteins were immunoprecipitated with anti-phosphotyrosine antibodies. In control CHO-T cells, stimulation with insulin resulted in a dose-dependent increase in PI 3-kinase activity. Maximal activation was 80-fold compared with unstimulated cells. Preincubation of the cells with CP (1 μM) or PI0 (25 μM) potentiated insulin activation of PI 3-kinase activity 2–3-fold (Fig. 3). This potentiation was found to be dependent upon the dose of CP used in the preincubation (Fig. 4A). Furthermore, the effect of CP on PI 3-kinase activity was apparent after pretreating the cells for 1 h prior to insulin stimulation and the maximal effect maintained through a 5-h preincubation period (Fig. 4B).

**Marine L6 muscle cells** possess many of the properties of skeletal muscle tissue when differentiated into myotubes (34). PI 3-kinase activity was found to be 4–5-fold greater in insulin-stimulated myotubes than in unstimulated cells (Fig. 5). Furthermore, CP potentiated insulin’s effect 2–3-fold (Fig. 5). This last result indicates that thiazolidinedione derivatives augment insulin activation of PI 3-kinase not only in cells overexpressing the insulin receptor but also in a cellular model of insulin-responsive tissue.

**Binding of PI 3-Kinase to Phosphotyrosine Proteins and IRS-1 Tyrosine Phosphorylation—**Upon stimulation of cells with insulin, PI 3-kinase becomes activated by binding to tyrosine-phosphorylated IRS-1, a major substrate of the IR tyrosine kinase (8). To examine the effects of thiazolidinedione-derived compounds on the association of PI 3-kinase with IRS-1 and tyrosine phosphorylation of IRS-1, CHO-T cells were preincubated with CP, AD, or no additions and then incubated in the absence or presence of insulin. The cells were lysed, and solubilized proteins were subjected to immunoprecipitation with anti-phosphotyrosine antibodies. The lysates and the immunoprecipitated proteins were fractionated by SDS-PAGE. Immunoblot analysis was performed utilizing monoclonal anti-phosphotyrosine antibodies (PY20). Molecular masses (in kDa) of marker proteins and the band corresponding to the β subunit of the insulin receptor (IRβ) are indicated.

**Fig. 1. $^{125}$I-Labeled insulin binding to CHO-T cells.** Cells were preincubated for 24 h at 37 °C with 25 μg PI0 (△), 1 μg CP (○), or with no additions (□). They were then incubated for 16 h at 4 °C with $^{125}$I-insulin in the presence of the indicated concentrations of unlabeled insulin. Subsequently, the cells were washed, and the $^{125}$I-insulin bound to the cells was quantified. Results are the means of triplicate determinations.

**Fig. 2. Insulin receptor tyrosine kinase and autophosphorylation in CHO-T cells.** A. control CHO (●), control CHO-T (○), and CHO-T cells treated for 5 h at 37 °C with 25 μg PI0 (△) or 1 μg CP (□) were stimulated with the indicated concentrations of insulin for 10 min at 37 °C. Cells were then lysed, and the receptors were captured on microtiter wells coated with monoclonal antibody against IR. IR tyrosine kinase activity was assayed by measuring $^{32}$P incorporation into the exogenous substrate poly(Glu-Tyr) (4:1). In parallel wells, the amount of captured IR was determined by $^{125}$I-insulin binding and used in normalization of the activity data. Results shown are means ± S.E. for triplicate determinations. B. CHO-T (○) or CHO-T pretreated for 5 h with CP (1 μM) or AD (1 μM) were incubated for 10 min at 37 °C with either buffer or 100 nM insulin as indicated. The cells were lysed, and solubilized proteins were separated by SDS-PAGE. Immunoblot analysis was performed utilizing monoclonal anti-phosphotyrosine antibodies (PY20). Molecular masses (in kDa) of marker proteins and the band corresponding to the β subunit of the insulin receptor (IRβ) are indicated.
the indicated concentrations of insulin for 5 min at 37 °C. PI 3-kinase activity was measured in anti-phosphotyrosine immunoprecipitates using PI as a substrate. A, phosphorimage of the [32P]phosphate-labeled PI 3-phosphate products (PIP). The positions of the origin of sampling (Ori) and PI 3-phosphate are indicated. B, quantification of the effect of CP and PIO on insulin stimulation of PI 3-kinase. The radioactivities associated with the spots on the thin-layer chromatography plates corresponding to PI 3-phosphate were determined by scintillation counting (control CHO; ○, control CHO-T; □, CHO-T treated with CP; ▲, CHO-T treated with PIO). Results shown are means ± S.E. of triplicate determinations.

Fig. 4. Effect of compound concentration and preincubation time on PI 3-kinase activation. A, CHO-T cells were preincubated for 5 h at 37 °C with the indicated concentrations of CP and then treated with 100 nM insulin at 37 °C for 5 min; B, cells were preincubated with 1 μM CP at 37 °C for the indicated periods of time and then treated with 100 nM insulin at 37 °C for 5 min. PI 3-kinase activity was determined as described in the legend of Fig. 3. 

In recent years, extensive studies on the insulin signal transduction pathways have led to the identification of an important endogenous IR tyrosine kinase substrate, IRS-1 (8). Insulin has also been shown to activate PI 3-kinase in cultured cells (14, 15) and in animals (17, 18). Further evidence supporting a role for PI 3-kinase in the insulin transduction pathways comes from experiments demonstrating that insulin stimulation of the kinase is attenuated in tissue from animals made insulin resistant by chemical treatment (38, 39) and in a genetic animal model of insulin resistance (40). The activation of PI 3-kinase apparently results from the binding of its p85 regulatory subunit SH2 domain to phosphotyrosines within several IRS-1 YMXM sequence motifs (10–13).

In the present study, we have investigated the effects of a novel class of insulin sensitizing agents, thiazolidinedione derivatives, on multiple steps in the insulin signal transduction pathway. Novel classes of agents that sensitize insulin resistance, such as compound 1, may provide important therapeutic leads for individuals suffering from the devastating increas
pathways. We demonstrated that these compounds enhance insulin activation of PI 3-kinase in CHO cells overexpressing the human insulin receptor (Fig. 3) and that the increments in the insulin-stimulated PI 3-kinase activity closely correlate with the increased amounts of enzyme associated with tyrosine-phosphorylated proteins (Fig. 6). Presumably, the major interaction being augmented by the thiazolidinedione derivatives is between the kinase and tyrosine-phosphorylated IRS-1. This potentiation appears to be both dose- and time-dependent (Fig. 4, a and b). The compounds exerted a similar activating effect on PI 3-kinase in L6 myotubes, a model of insulin-responsive skeletal muscle (Fig. 5). This last result demonstrates that the insulin potentiation effect of thiazolidinedione derivatives is not an artifact arising from the high level of insulin receptors expressed by CHO-T cells but may be indicative of their primary in vivo activity.

The mechanism(s) by which the thiazolidinedione derivatives potentiate insulin activation of PI 3-kinase is not yet entirely clear. They appear to act on postreceptor steps since they did not alter insulin binding to its receptor (Fig. 1) and failed to enhance the tyrosine kinase activity of the receptor when measured using an exogenous substrate (Fig. 2). In addition, we did not detect any gross alterations in tyrosine phosphorylation of IRS-1 by immunoblot analysis (Fig. 3). However, it is possible that this technique does not possess the sensitivity needed to detect changes at a limited number of phosphorylation sites. IRS-1 contains over 20 putative tyrosine and 30 potential serine/threonine phosphorylation sites. The latter contains regions with homology to consensus sequences for casein kinase II, protein kinase C, MAP kinase, cdc2, and cAMP- and cGMP-dependent protein kinase phosphorylation sites (41). Changes in the level of phosphorylation at any of these sites could potentially alter the ability of IRS-1 to bind and
activate PI 3-kinase.

IRS-1 has been shown to be involved in insulin-stimulated mitogenic signaling (42). The fact that the anti-diabetic agents did not alter insulin-induced DNA synthesis in CHO-T cells (Fig. 9) suggests that their effect on PI 3-kinase is relatively specific. In agreement with this observation, the compounds were unable to augment insulin activation of MAP kinase.

The binding of the p85 subunit of PI 3-kinase to IRS-1 appears to result in conformational changes in the enzyme (43, 44). Such alterations may serve to activate the enzyme and may be potentiated by the thiazolidinedione derivatives. It has also been demonstrated that PI 3-kinase is a dual-specificity enzyme containing an intrinsic serine kinase activity that phosphorylates Ser610 of the p85 subunit. This phosphorylation modulates the enzyme's affinity for phosphotyrosine residues (46, 47). Therefore, it is apparent that the activity of PI 3-kinase is modulated by a number of complex processes including protein-protein interactions, induced conformational changes, and alterations in its phosphorylation state. These events may not be independent but may modify each other through “cross-talk” during the activation process. The thiazolidinedione derivatives may potentiate any or all of these activation processes, perhaps as an affecter of the appropriate kinase or phosphatase. Alternatively, the compounds may promote association of PI 3-kinase with another phosphotyrosine protein and, thus, potentiate insulin activation of the enzyme.

The primary physiological action of PI 3-kinase has not yet been delineated. However, based upon the sequence homology of its p110 subunit to the yeast VPS34 gene product, the kinase may be involved in mediating intracellular protein sorting (48). Studies using growth factor receptors containing PI 3-kinase binding site mutations support the conclusion that the enzyme plays a role in mediating receptor endocytosis and intracellular protein trafficking (49). Further work is necessary to define the role of PI 3-kinase in the insulin signal transduction pathways regulating metabolism and mitogenesis.

In summary, insulin resistance of peripheral tissue is a major pathological component of NIDDM. The thiazolidinedione-derived anti-diabetic agents lower plasma glucose and triglyceride levels in animal models and humans suffering from obesity and NIDDM. These beneficial effects are apparently the result of the compounds acting as insulin sensitizers in insulin-responsive tissue. Here, we have demonstrated that the thiazolidinedione derivatives potentiate insulin stimulation of PI 3-kinase. Intriguingly, the thiazolidinediones appear to be rather selective in their insulin-dependent activation of PI 3-kinase, since other insulin-dependent events such as activation of MAP kinase and mitogenesis were unaffected. This finding unveils a putative molecular mechanism of action for this class of anti-diabetic agents and demonstrates a potential use for these compounds as tools to study the insulin signal transduction pathways.

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REFERENCES

1. White, M. F., and Kahn, C. R. (1994) J. Biol. Chem. 269, 1-4
2. Rosenson, O., Herrera, P., Olows, T., Petrozziello, L., and Cobb, M. H. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 3327-3340
3. Yu, J.-T., and Czech, M. P. (1986) J. Biol. Chem. 261, 4715-4722
4. Kupfer, H., Pfeiffer, G. B., Hell, M., and Oseiski, J. M. (1986) J. Biol. Chem. 261, 4691-4697
5. Herrera, R., Lebwohl, D., de Herreras, A. G., Kallen, R. G., and Rosen, O. M. (1986) J. Biol. Chem. 262, 5590-5596
6. Cobb, M. H., Sang, B.-C., Gonzalez, R., Goldsmith, E., and Ellis, L. (1989) J. Biol. Chem. 264, 18701-18706
7. Enggert, C., Lauer, C., Morgan, D. O., Ederly, M., Roth, R. A., and Rutter, W. J. (1988) Cell 50, 721-726
8. Sun, X.-J., Rothberg, P., Kahn, C. R., Backer, M. F., Azaki, E., Wilden, P. A., Cahlill, D., Goldstein, B. J., and White, M. F. (1991) Nature 348, 73-77
9. Cantley, L. C., Auger, K. R., Carpenter, C., Duckworth, B., Gruen, A., Kapeler, R., and Sultoff, S. (1991) Cell 45, 321-352
10. Myers, G. M., Backer, J. M., Sun, S. J., Shoebone, S. H., Pechilon, J., Voilund, M., Schaffner, A., and White, M. P. (1992) Proc. Natl. Acad. Sci. U. S. A. 90, 10350-10354
11. Folli, F., Sald, M. J. A., Backer, J. M., and Kahn, C. R. (1992) J. Biol. Chem. 267, 22171-22177
12. Giorgi, C., Ciotti, C., Kolczak, A., Gallant, S. A., and Van OBergen, B. (1993) J. Biol. Chem. 268, 7356-7364
13. Backer, J. M., Myers, M. G., Jr., Sun, X. J., Chin, D. J., Shoebone, S. E., Miura, M., and White, M. F. J. Biol. Chem. 268, 8204-8212
14. Ruderman, N. B., Kapeler, R., White, M. F., and Cantley, L. C. (1990) Proc. Natl. Acad. Sci. U. S. A. 87, 1411-1415
15. Endemmen, G., Yonezawa, K., and Roth, R. A. (1990) J. Biol. Chem. 265, 390-400
16. Kagi, K. S., and Ruderman, N. B. (1993) J. Biol. Chem. 268, 4391-4398
17. Kelly, K. L., Ruderman, N. B., and Chen, K. S. (1997) J. Biol. Chem. 267, 3423-3428
18. Hackett, Y., Teshar, E., Nadjiv, O., Rothenberg, P., Roberts, C. T., Jr., LeRoith, D., Yarden, R., and Zick, Y. (1992) J. Biol. Chem. 267, 17483-17486
19. DeFronzo, R. A., Bonadonna, R. C., and Ferrannini, E. (1999) Diabetes Care 15, 318-328
20. Breswill, R., and Johnson, D. (1992) Diabetes Care 15, 792-805
21. Chang, A. Y., Wisby, B. M., Gilchirst, B. J., and Pate, T., and Diaci, A. R. (1993) Diabetes 32, 830-838
22. Chang, A. Y., Gilchrest, B. J., and Wisby, B. M. (1985) Diabetologia 26, 514-520
23. Fujita, T., Sugiyama, Y., Takemoto, S., Sibata, T., Kawamata, S., Iwatek, H., and Suzuki, Z. (1983) Diabetes 32, 843-849
24. Sugiyama, T., Takemori, S., Shimura, Y., Iida, H., and Fujita, T. (1990) Arzneim-forehch. 40, 263-267
25. Weinsteins, S. P., Holand, A. O'Brye, E., and Rapp, R. S. (1993) Metabolism 42, 1585-1589
26. Blackmore, P. F., McPherson, R. K., and Stevenson, R. W. (1993) Metabolism 42, 1585-1589
27. Kozak, T., Tanu, Y., Kosaka, K., Kabe, Y., Yamanouchi, T., Kusagawa, M., Kajinuma, H., Akanuma, Y., Yoshida, S., Shige, Y., and Baba, S. (1991) Diabetes Res. Clin. Practice 11, 147-154
28. Sater, S. L., Nolan, J. S., Wallace, P., Gombiner, B., and Oseiski, J. M. (1992) Diabetes Care 15, 193-203
29. Harlow, E., and Lane, D. (1988) Antibodies: A Laboratory Manual Cold Spring Harbor Library. Cold Spring Harbor, NY.
30. Hayes, N., Biswas, C., Strat, E., and Brown, J. J. (1992) J. Biol. Chem. 268, 881-887
31. Zhang, B., Tavare, J. M., Ellis, L., and Roth, R. A. (1991) J. Biol. Chem. 266, 990-996
32. Whitman, M., Kaplan, D. B., Schaffhausen, B., Cantley, L., and Roberts, T. M. (1994) Nature 313, 517-525
33. Moller, C., Hanaus, A., Emberg, B., Lohse, P. E., and Norsted, O. (1993) J. Biol. Chem. 267, 53403-53408
34. Klap, A., and Marette, A. (1992) J. Cell Biol. 48, 741-746
35. Sturgill, T. W., and Ray, L. B. (1986) Biochem. Biophys. Res. Commun. 138, 565-571
36. Ray, L. B., and Sturgill, T. W. (1987) Proc. Natl. Acad. Sci. U. S. A. 84, 1502-1506
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37. Tobe, K., Kadowaki, T., Haro, K., Gotch, Y., Kosako, H., Matsuda, S., Tamegato, H., Ueki, D., Akamine, Y., Nishida, E., and Yazaki, Y. (1992) J. Biol. Chem. 267, 21069-21077
38. Heyrick, S. J., Julienne, D., Gautier, N., Tanti, J.-F., Giorgetti, S., Van Obberghen, E., and Le Marchand-Brustel, Y. (1993) J. Clin. Invest. 91, 1358-1366
39. Saad, M. J. A., Folli, F., Kahlo, J. A., and Kahn, C. R. (1993) J. Clin. Invest. 92, 2065-2072
40. Folli, F., Saad, M. J. A., Backer, J. M., and Kahn, C. R. (1993) J. Clin. Invest. 92, 1757-1764
41. Myers, M. G., and White, M. F. (1993) Diabetes 42, 643-650
42. Waters, S. B., Yamauchi, D., and Pessin, J. E. (1993) J. Biol. Chem. 268, 22231-22234
43. Panayotou, G., Bax, B., Gout, I., Federwisch, M., Wroblewski, B., Dhan, R., Fry, M. J., Blundell, T. L., Wollmer, A., and Waterfield, M. D. (1992) EMBO J. 11, 4261-4272
44. Carpeneix, C. L., Auger, K. R., Duckworth, B. C., Yoakim, M., Schaffhausen, B., and Cantley, L. C. (1993) Mol. Cell. Biol. 13, 1657-1665
45. Dhand, R., Hiles, I., Panayotou, G., Roche, S., Fry, M. J., Gout, I., Totty, N., Truong, O., Vicente, P., Yonezawa, K., Kasuga, M., Courtnedge, S. A., and Waterfield, M. D. (1994) EMBO J. 13, 522-533
46. Hayashi, H., Kamohara, S., Nishioka, Y., Kanai, F., Miyake, N., Fukui, Y., Shibasaki, F., Takenawa, T., and Ebina, Y. (1992) J. Biol. Chem. 267, 22575-22580
47. Hayashi, H., Nishioka, Y., Kamohara, S., Kanai, F., Ishii, K., Fukui, Y., Shih-Pao, F., Takenawa, T., Kido, H., Katsumata, N., and Ebina, Y. (1993) J. Biol. Chem. 268, 7107-7117
48. Schu, P. V., Takegawa, K., Fry, M. J., Stack, J. H., Waterfield, M. D., and Emr, S. D. (1993) Science 260, 88-91
49. Joly, M., Kazlauske, A., Fay, F. S., and Corvea, S. (1994) Science 263, 674-687