0022-3565/03/3043-994–1002$7.00
THE JOURNAL OF PHARMACOLOGY AND EXPERIMENTAL THERAPEUTICS
Vol. 304, No. 3
Copyright © 2003 by The American Society for Pharmacology and Experimental Therapeutics
44115/1042024
JPET 304:994–1002, 2003
Printed in U.S.A.

Distinct Effects of Ketone Bodies on Down-Regulation of Cell Surface Insulin Receptor and Insulin Receptor Substrate-1 Phosphorylation in Adrenal Chromaffin Cells

HIROKI YOKOO, TOMOKAZU SAITOH, SEIJI SHIRAISHI, TOSHIHIKO YANAGITA, TAKASHI SUGANO, SHIN-ICHI MINAMI, HIDEYUKI KOBAYASHI, and AKIHIKO WADA

Department of Pharmacology, Miyazaki Medical College, Miyazaki, Japan

Received September 5, 2002; accepted November 11, 2002

ABSTRACT

Treatment (≧24 h) of cultured bovine adrenal chromaffin cells with ketoacidosis-related concentrations (≧3 mM) of acetoacetate (but not β-hydroxybutyrate, acetone, and acidic medium) caused a time- and concentration-dependent reduction of cell surface ^{125}I-insulin binding by ~38%, with no change in the K_d value. The reduction of ^{125}I-insulin binding returned to control nontreated level at 24 h after the washout of acetoacetate-treated cells. Acetoacetate did not increase the internalization rate of cell surface insulin receptor (IR), as measured in the presence of brefeldin A, an inhibitor of cell surface vesicular exit from the *trans*-Golgi network. Acetoacetate (10 mM for 24 h) lowered cellular levels of the immunoreactive IR precursor molecule (~190 kDa) and IR by 22 and 28%, respectively. Acetoacetate decreased IR mRNA levels by ~23% as early as 6 h, producing their maximum plateau reduction at 12 and 24 h. The half-life of IR mRNA was shortened by acetoacetate from 13.6 to 9.5 h. Immunoprecipitation followed by immunoblot analysis revealed that insulin-induced (100 nM for 10 min) tyrosine-phosphorylation of insulin receptor substrate-1 (IRS-1) was attenuated by 56% in acetoacetate-treated cells, with no change in IRS-1 level. These results suggest that chronic treatment with acetoacetate selectively down-regulated the density of cell surface functional IR via lowering IR mRNA levels and IR synthesis, thereby retarding insulin-induced activation of IRS-1.

Physiological hyperketonemia occurs quite readily via "Randle's glucose-fatty acid cycle" as a compensatory defensive response against fasting, particularly in the neonate and pregnancy, occasionally developing into frank ketoacidosis (Féry and Balasse, 1985; Laffel, 1999). Also, hyperketonemia is brought about during prolonged exercise and high-fat diet in normal individuals. In patients with congenital enzyme defects unable to catalyze hepatic mitochondrial synthesis of ketone bodies, even short-term fasting causes hypoketotic hypoglycemia, increased levels of plasma free fatty acids (FFA), and childhood sudden death because of their inability to oxidize FFA into ketone bodies (Hashimoto et al., 2000). In contrast, diabetic ketoacidosis due to the defective insulin secretion and insulin resistance is a life-threatening state. In patients with diabetes mellitus, obesity, and atherosclerotic vascular diseases, the increased levels of plasma FFA are linked to the insulin-resistant state in these diseases (Boden et al., 2001) because FFA interfere with insulin's intracellular signaling (Patti, 1999). Density of cell surface insulin receptors (IR), a member of the receptor tyrosine kinase family, is a key determinant in regulating the strength of insulin's acute and chronic pleiotropic effects, such as the metabolic and neurotropic effects (Dikic et al., 1994). In pancreatic β-cells, evidence has emerged that IR regulate secretion (Aspinwall et al., 2000) and synthesis (Leibiger et al., 1998; Jackerott et al., 2001) of insulin via an autocrine manner. Neonatal mouse lacking IR rapidly developed into the lethal diabetic ketoacidosis (Jackerott et al., 2001).

IR consist of two extracellular α-subunits (~135 kDa) and two transmembrane β-subunits (~95 kDa), which are encoded by the same gene and derived from the single chain IR precursor molecule (~190 kDa) (Cheatham and Kahn, 1995). IR precursor undergoes cotranslational glycosylation, intrachain disulfide-bond formation/isomerization, and disulfide-linked homodimerization at the endoplasmic reticulum (ER). The homodimeric IR precursor is proteolytically processed into the disulfide-linked $\alpha_2\beta_2$ complex at the *trans*-Golgi network, which is transported to the plasma membrane via an

This study was supported in part by a grant-in-aid for the 21st Century COE (Centers of Excellence) Program (Life Science) from the Ministry of Education, Culture, Sports, Science and Technology, Japan.

Article, publication date, and citation information can be found at http://jpet.aspetjournals.org.

DOI: 10.1124/jpet.102.044115.

ABBREVIATIONS: FFA, free fatty acids; IR, insulin receptors; ER, endoplasmic reticulum; IRS-1, insulin receptor substrate-1; cPGI$_2$, 6α-carba-prostaglandin I$_2$; GAPDH, glyceraldehyde-3-phosphate-dehydrogenase; DMSO, dimethyl sulfoxide; KRP, Krebs-Ringer phosphate; PAGE, polyacrylamide gel electrophoresis; SSC, saline-sodium citrate; PPAR, peroxisome proliferator-activated receptors; kb, kilobase.

as yet unidentified mechanism (Cheatham and Kahn, 1995). Binding of insulin to the α-subunit causes autophosphorylation of the β-subunit, leading to the endocytic internalization of IR via clathrin-coated vesicles. IR internalization may trigger phosphorylation of insulin receptor substrate-1 (IRS-1) at the multiple tyrosine residues, which serve as binding sites for various signaling molecules containing the Src homology-2 domain, thus triggering insulin's pleiotropic effects (Cheatham and Kahn, 1995).

In adrenal chromaffin cells (embryologically derived from the neural crest), IR play pivotal roles, such as up-regulation of cell surface voltage-dependent Na^+ channels (Yamamoto et al., 1996) and enhancement of voltage-dependent Ca^{2+} channel gating and of exocytic secretion of catecholamines (Yamamoto et al., 1996), as well as increased synthesis of various bioactive peptides (e.g., enkephalin) contained within chromaffin granules (Wilson et al., 1985). Our previous study showed that chaperone function of the heat shock protein 90-kDa family was indispensable to the homodimerization of monomeric IR precursor at the ER (Saitoh et al., 2002). Protein kinase C-α increased IR mRNA and protein levels, thus causing up-regulation of cell surface IR (Yamamoto et al., 2000). Also, peptidyl prolyl *cis-trans*-isomerase activity of immunophilins (Shiraishi et al., 2000) and Ca^{2+}-ATPase activity of the ER (Shiraishi et al., 2001) promoted cell surface externalization of IR from the *trans*-Golgi network. In the present study, ^{125}I-insulin binding, immunoblot, and Northern blot analyses show that chronic treatment with acetoacetate (but not β-hydroxybutyrate, acetone, and acidic medium) selectively down-regulated cell surface expression of IR via lowering IR mRNA levels and IR synthesis, thereby attenuating insulin-induced tyrosine-phosphorylation of IRS-1.

Materials and Methods

Materials. Eagle's minimum essential medium was from Nissui Seiyaku (Tokyo, Japan). Calf serum, phenylmethylsulfonyl fluoride, leupeptin, aprotinin, sodium orthovanadate, sodium fluoride, Nonidet P-40, Tween 20, and NaN_3 were from Nacalai Tesque (Kyoto, Japan). Acetoacetate, *dl*-β-hydroxybutyrate, clofibrate, 6α-carba-prostaglandin I_2 ($cPGI_2$), oleic acid, arachidonic acid, eicosapentaenoic acid, brefeldin A, and actinomycin D were from Sigma-Aldrich (St. Louis, MO). Acetone was from Wako Pure Chemicals (Osaka, Japan). Troglitazone was kindly donated from Sankyo (Tokyo, Japan). Rabbit polyclonal antibody against the human IR β-subunit was from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit polyclonal IRS-1 antibody was from Upstate Biotechnology (Lake Placid, NY). Mouse monoclonal phosphotyrosine-specific antibody (PY-20) was from BD PharMingen (San Diego, CA). Protein A-agarose and Oligotex-dT30<Super> were from Nippon Roche (Tokyo, Japan). TRIzol reagent was from Invitrogen (Carlsbad, CA). The BcaBEST labeling kit and Noninterfering Protein Assay kit were from Takara (Kyoto, Japan). ^{125}I-Labeled donkey anti-rabbit IgG, ^{125}I-insulin (~2000 Ci/mmol), and [α-^{32}P]dCTP (>4000 Ci/mmol) were from Amersham Biosciences (Piscataway, NJ). ^{125}I-Insulin was diluted with nonradioactive human insulin Humulin R (Eli Lilly, Kobe, Japan), and ^{125}I-insulin (3.125 Ci/mmol) was used for the ^{125}I-insulin binding assay. cDNA for human glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) was from CLONTECH Laboratories (Palo Alto, CA). Plasmid containing human IR cDNA (pSELECT HIR) was generously donated from Drs. Graeme Bell and Donald F. Steiner (Yamamoto et al., 2000).

Primary Culture of Adrenal Chromaffin Cells: Treatment with Test Compounds and Acidic Medium. Isolated bovine adrenal chromaffin cells were cultured (4×10^6 per dish, Falcon; 35-mm diameter) in Eagle's minimum essential medium containing 10% calf serum under 5% CO_2/95% air in a CO_2 incubator (Yamamoto et al., 1996, 2000). Three days (60–62 h) later, the cells were treated in the fresh culture medium with or without acetoacetate, β-hydroxybutyrate, acetone, clofibrate, $cPGI_2$, or troglitazone for up to 48 h or exposed to freshly prepared acidic culture medium (pH 6.9) for 24 h. The pH values of these test compound-containing media were 7.4, as measured by pH meter F-14 (Horiba, Kyoto, Japan). The acidic medium was prepared by adding 1 N HCl to the culture medium, its pH value being reconfirmed as 6.9 after the 24-h incubation period. Ketone bodies and other test compounds were dissolved in dimethyl sulfoxide (DMSO) and ethanol, respectively. The final concentrations (~0.2%) of DMSO and ethanol in the test medium did not affect ^{125}I-insulin binding capacity, IR precursor, and IR β-subunit levels, or the basal tyrosine-phosphorylation level of IRS-1. The culture medium contained 3 μM cytosine arabinoside to suppress the proliferation of nonchromaffin cells; when chromaffin cells were further purified by differential plating (Yamamoto et al., 1996, 2000), ^{125}I-insulin binding was similar between purified and conventional chromaffin cells. Also, acetoacetate treatment (10 mM for 24 h) decreased ^{125}I-insulin binding by 27 and 28% in purified and conventional chromaffin cells compared with nontreated cells in each cell group.

^{125}I-Insulin Binding. Cells were washed with ice-cold Krebs-Ringer phosphate (KRP) buffer (154 mM NaCl, 5.6 mM KCl, 1.1 mM $MgSO_4$, 2.2 mM $CaCl_2$, 0.85 mM NaH_2PO_4, 2.15 mM Na_2HPO_4, 5 mM glucose, and 0.5% bovine serum albumin, pH 7.4) and incubated with 0.025 to 10 nM ^{125}I-insulin in 1 ml of KRP buffer at 4°C for 6 h in the absence (total binding) and presence (nonspecific binding) of 1 μM unlabeled insulin. The cells were immediately washed, solubilized in 0.2 M NaOH, and counted for radioactivity. Specific binding was calculated as the total binding minus nonspecific binding. The B_{max} and K_d values of ^{125}I-insulin binding correspond to the binding of ^{125}I-insulin to IR (but not insulin-like growth factor receptors), as reported previously (Yamamoto et al., 1996, 2000; Shiraishi et al., 2000, 2001). ^{125}I-Insulin binding represents cell surface (but not internalized) IR because ^{125}I-insulin associated with chromaffin cells were completely removed by washing the cells with ice-cold acidic (pH 4.0) KRP buffer twice, each for 7 min, as reported previously (Yamamoto et al., 2000).

Western Blot Analysis of IR and the IR Precursor Molecule. Cells were washed with ice-cold Ca^{2+}-free phosphate-buffered saline and solubilized in 500 μl of 2× SDS electrophoresis sample buffer [125 mM Tris-HCl (pH 6.8), 20% glycerol, 10% 2-mercaptoethanol, and 4% SDS] at 98°C for 3 min. Total quantities of cellular proteins, as measured by the Noninterfering Protein Assay kit, were not changed between nontreated and ketone body-treated or acidic medium-treated cells. The same amounts of proteins (7.0–7.5 μg/lane) were separated by SDS-7.5% polyacrylamide gel electrophoresis (PAGE) and transferred onto a nitrocellulose membrane; it was preincubated with 5% dry milk in phosphate-buffered saline and reacted overnight at 4°C with rabbit antibody against the C-terminal amino acid sequence (1365–1382) of the IR β-subunit (Cheatham and Kahn, 1995; Shiraishi et al., 2000, 2001; Yamamoto et al., 2000; Saitoh et al., 2002). After repeated washings, the immunoreactive bands were labeled with ^{125}I-anti-rabbit IgG (1:1000) and analyzed by a Bioimage BAS 2000 analyzer (Fuji Film, Tokyo, Japan).

Northern Blot Analysis of IR mRNA Levels. Total cellular RNA was isolated from cells by acid guanidine-thiocyanate-phenol-chloroform extraction using TRIzol reagent. poly(A)$^+$RNA was purified by Oligotex-dT30<Super>, electrophoresed on 1% agarose gel containing 6.3% formaldehyde in buffer [40 mM 3-(*N*-morpholino) propanesulfonic acid (pH 7.2), 0.5 mM EDTA, and 5 mM sodium citrate], transferred to a nylon membrane (Hybond-N; Amersham Biosciences) in 20× saline-sodium citrate (SSC; 1× SSC = 0.15 M NaCl and 0.015 M sodium citrate) overnight and cross-linked using a UV cross-linker (Funakoshi, Tokyo, Japan). The IR cDNA fragment (nucleotides 1–4608), obtained by digestion of pSELECT HIR with *Sal*I, and GAPDH cDNA (1.1 kilobase pairs) were labeled with [α-^{32}P]dCTP using the BcaBEST labeling kit (Yamamoto et al., 2000; Saitoh et al., 2002). The membrane was prehybridized at 42°C in 6× SSC, 10× Denhardt's solution (2% bovine serum albumin fraction V, 2% polyvinylpyrrolidone, and 2% Ficoll 400), 50% formamide, 0.5%

SDS, and 50 μg/ml salmon sperm DNA and then hybridized with the IR probe under the same condition for 18 h. It was washed at 55°C in 2×, 1×, and 0.2× SSC containing 0.1% SDS, each for 30 min twice, and subjected to autoradiography. The same membrane was hybridized with the GAPDH probe after it was thoroughly washed in 0.1% SDS at 100°C to remove the IR probe. The autoradiogram was quantified by a Bioimage BAS 2000 analyzer.

Immunoprecipitation, PAGE, and Immunoblot Analysis of IRS-1 and Tyrosine-Phosphorylated IRS-1. Cells were washed, solubilized at 4°C for 15 min in 1 ml of lysis buffer [50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 1 mM phenylmethylsulfonyl fluoride, 10 mM EDTA, 20 μg/ml aprotinin, and 10 μg/ml leupeptin], and centrifuged at 12,000*g* for 10 min at 4°C. The supernatant was reacted with protein A-agarose for 1 h at 4°C and centrifuged. Proteins in the supernatant were immunoprecipitated with IRS-1 antibody for 2 h at 4°C and then with protein A-agarose for 1 h. The immunoprecipitate was washed three times with the lysis buffer by repeated resuspension and centrifugation, solubilized in 25 μl of 2× SDS electrophoresis sample buffer at 98°C, and centrifuged to remove protein A-agarose. Proteins in the supernatant were size-fractionated by SDS-PAGE and transferred to membrane for immunoblot analysis of IRS-1 level. To measure insulin-induced tyrosine-phosphorylation of IRS-1, cells were washed with KRP buffer and incubated at 37°C with or without 100 nM insulin for 10 min in 1 ml of KRP buffer; cells were solubilized in the lysis buffer containing 100 mM NaF and 10 mM Na_3VO_4 and subjected to immunoprecipitation with IRS-1 antibody.

For immunoblot analysis, the membrane was preincubated with Tween-Tris-buffered solution [10 mM Tris-HCl (pH 7.4), 150 mM NaCl, and 0.1% Tween 20] containing 1% bovine serum albumin and 0.05% NaN_3 and reacted overnight at 4°C with IRS-1 antibody or phosphotyrosine-specific antibody (Saitoh et al., 2002). After repeated washings with Tween-Tris-buffered solution, the immunoreactive bands were labeled with ^{125}I-anti-rabbit IgG and analyzed by a Bioimage BAS 2000 analyzer.

Statistical Methods. ^{125}I-Insulin binding was performed in triplicate, and all experiments were repeated at least three times (mean ± S.E.M.). Significance ($p < 0.05$) was determined by one-way or two-way analysis of variance with post hoc mean comparison by Newman-Keuls multiple range test. Student's *t* test was used when two means of a group were compared.

Results

Reduction of Cell Surface ^{125}I-Insulin Binding by Chronic Treatment with Acetoacetate: No Effect of β-Hydroxybutyrate, Acetone, Acidic Medium, Clofibrate, cPGI$_2$, and Troglitazone. It has been shown that in patients with noninsulin-dependent diabetes mellitus under good nutritional status, plasma concentrations of acetoacetate and β-hydroxybutyrate ranged between 0.25 to 3.05 and 0.66 to 10.23 mM, respectively (Féry and Balasse, 1985). Total plasma ketone bodies (acetoacetate plus β-hydroxybutyrate) are >1 mM in hyperketonemia and >3 mM in ketoacidosis, developing to >25 mM with the preferential increase of β-hydroxybutyrate in diabetic ketoacidosis (Laffel, 1999). As shown in Fig. 1A, treatment of adrenal chromaffin cells with 10 mM acetoacetate for 24 h decreased cell surface ^{125}I-insulin binding capacity by 28%, whereas the same treatment with 10 mM β-hydroxybutyrate, 100 mM acetone, or acidic medium (pH 6.9) had no effect. Figure 1B shows that the decreasing effect of acetoacetate on ^{125}I-insulin binding became evident between 12 and 24 h, the reduction attaining to 28, 36, and 38% at 24, 36, and 48 h, respectively. Cells were treated with acetoacetate for the first 24 h, then washed (Fig. 1B, arrow), and incubated in the absence of acetoacetate for up to 48 h; ^{125}I-insulin binding gradually returned to the control nontreated level between 24 and 48 h. Figure 1C shows that acetoacetate decreased ^{125}I-insulin binding in a concentration-dependent manner between 3 and 60 mM. Rosenthal plot analysis (Fig. 1D) revealed that acetoacetate (10 mM for 24 h) decreased the B_{max} values, with a statistical significance ($P < 0.05$) from 115.5 ± 4.2 to 83.2 ± 3.8 fmol/4 × 10^6 cells, without altering the K_d values (3.5 ± 0.3 nM, nontreated cells; 3.4 ± 0.3 nM, acetoacetate-treated cells; $n = 5$).

Evidence has emerged that polyunsaturated FFA and FFA-derived eicosanoids bind to peroxisome proliferator-activated receptors (PPAR), a family of transcription factors, and regulate expression of genes involved in glucose and lipid homeostasis (Willson et al., 2001). Although it is unknown whether acetoacetate binds to PPAR, we raised the question whether ligands for PPAR decrease ^{125}I-insulin binding. Treatment of adrenal chromaffin cells for 24 h with 300 μM clofibrate (PPARα ligand), 20 μM cPGI$_2$ (PPARδ/β ligand), or 50 μM troglitazone (PPARγ ligand) did not alter ^{125}I-insulin binding (fmol/4 × 10^6 cells; 1.42 ± 0.08, nontreated cells; 1.47 ± 0.10, clofibrate-treated cells; 1.48 ± 0.04, cPGI$_2$-treated cells; 1.40 ± 0.06, troglitazone-treated cells; $n = 3$). Also, treatment of adrenal chromaffin cells for 24 h with polyunsaturated FFA (e.g., 10 mM oleic acid, 100 μM arachidonic acid, and 100 μM eicosapentaenoic acid) did not lower ^{125}I-insulin binding (authors' unpublished observation).

Internalization Rate of Cell Surface IR: No Effect of Chronic Treatment with Acetoacetate. By using brefeldin A, we examined whether acetoacetate may accelerate the internalization rate of cell surface IR. Brefeldin A is an inhibitor of guanine nucleotide-exchange protein of ADP-ribosylation factor 1, a monomeric GTPase. Previous studies showed that brefeldin A treatment (2.5–10 μg/ml for 2–36 h) of various intact cells blocks cell surface externalization from the *trans*-Golgi network of newly synthesized transferrin receptors, α_{1B}-adrenoceptors, renal epithelial sodium channels, and glucose transporter-4, whereas having no effect on ADP-ribosylation factor 6-catalyzed internalization of cell surface receptors and ion channels (Shiraishi et al., 2000, 2001; Yamamoto et al., 2000). In adrenal chromaffin cells, previous fluorescence study showed that brefeldin A treatment (0.28–2.8 μg/ml for 2 h) was sufficient to cause disassembly of the Golgi membrane in most (>90%) chromaffin cells (Xu and Tse, 1999).

Figure 2 shows that adrenal chromaffin cells were treated with or without 10 μg/ml brefeldin A and 10 mM acetoacetate for up to 18 h, and cell surface ^{125}I-insulin binding was assayed at the indicated times. In cells treated with brefeldin A alone, ^{125}I-insulin binding was progressively decreased in a time-dependent manner with a $t_{1/2}$ of 7.5 h, a value consistent with previous studies (Shiraishi et al., 2000, 2001). The coincident treatment with acetoacetate, however, did not augment the reduction to any more extent compared with brefeldin A treatment alone.

Cellular Levels of Immunoreactive IR β-Subunit and IR Precursor Molecule: Reduction by Chronic Treatment with Acetoacetate, but Not with β-Hydroxybutyrate, Acetone, and Acidic Medium. Western blot analysis (Fig. 3A) shows that the IR β-subunit antibody recognized single major (~95 kDa) and single minor (~190 kDa) bands, which is in agreement with the molecular sizes of the mature IR β-subunit and IR precursor molecule, respectively (Cheatham and Kahn, 1995; Shiraishi et al., 2000, 2001; Yamamoto et al., 2000; Saitoh et al., 2002). The antibody,

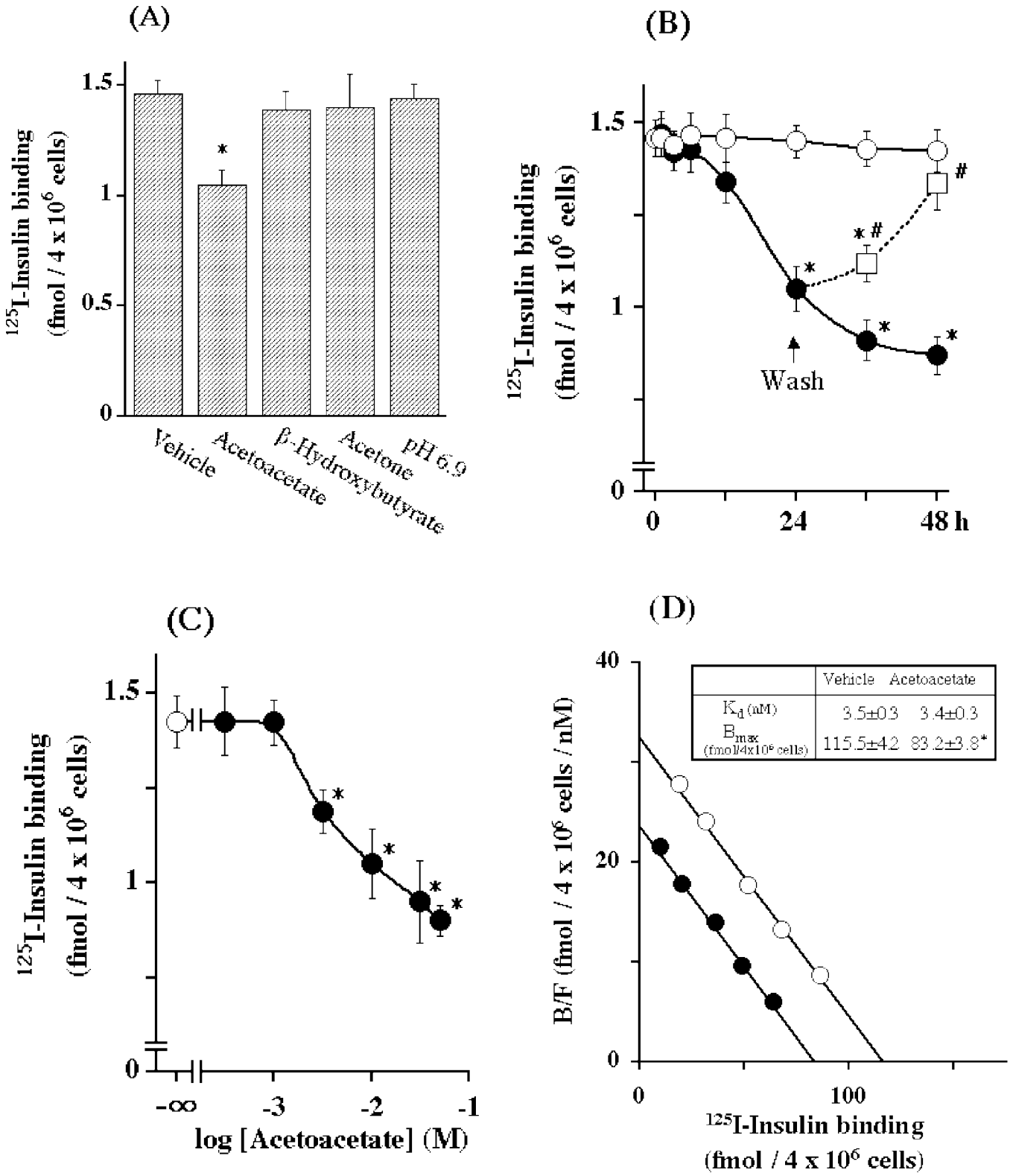

Fig. 1. Chronic treatment of adrenal chromaffin cells with acetoacetate, β-hydroxybutyrate, acetone, and acidic medium: their effects on cell surface ^{125}I-insulin binding. A, cells were treated without (vehicle) or with 10 mM acetoacetate, 10 mM β-hydroxybutyrate, 100 mM acetone or acidic medium (pH 6.9) for 24 h, and ^{125}I-insulin binding was assayed. B, cells were treated with (●) or without (○) 10 mM acetoacetate for up to 48 h and subjected to ^{125}I-insulin binding assay at the indicated times. In parallel study, cells were treated with 10 mM acetoacetate for the first 24 h, washed (indicated by arrow), then incubated in the absence (□) of acetoacetate, and subjected to ^{125}I-insulin binding assay at 36 and 48 h. C, cells were treated with (●) or without (○) 0.3 to 60 mM acetoacetate for 24 h and subjected to ^{125}I-insulin binding assay. Mean ± S.E.M. (n = 3). D, Rosenthal plot of ^{125}I-insulin binding to the cells treated with (●) or without (○) 10 mM acetoacetate for 24 h. The plot is typical from five separate experiments with similar results (inset). *, $P < 0.05$ compared with nontreated cells; #, $P < 0.05$ compared with acetoacetate-treated cells; B/F, bound/free.

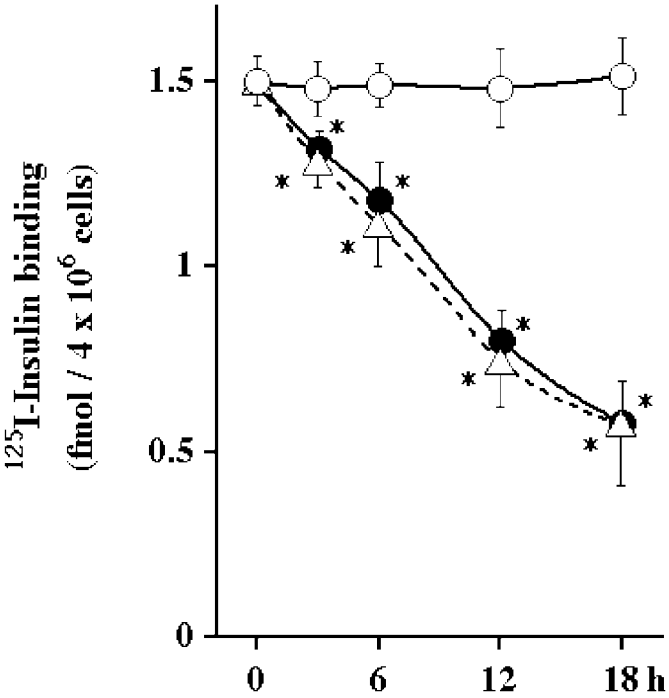

Fig. 2. Internalization of cell surface IR: no effect of acetoacetate treatment. In the absence (○) or presence (△, ●) of 10 μg/ml brefeldin A, cells were treated with (●) or without (△) 10 mM acetoacetate for up to 18 h; at each indicated incubation time, the remaining cell surface IR was measured by ^{125}I-insulin binding assay. Mean ± S.E.M. (n = 5). *, P < 0.05 compared with brefeldin A-nontreated cells.

when reacted with its immunogen before the immunoblot analysis, did not recognize these bands (data not shown). Quantification of these immunoreactive bands (Fig. 3B) shows that acetoacetate (10 mM for 24 h) lowered cellular levels of the IR β-subunit and IR precursor molecule by 28 and 22%, respectively. In contrast, the same 24-h treatment with 10 mM β-hydroxybutyrate, 100 mM acetone, or acidic medium (pH 6.9) had no effect on the IR β-subunit and IR precursor levels.

IR mRNA Levels: Reduction by Chronic Treatment with Acetoacetate. As shown in Fig. 4A, the IR cDNA probe hybridized to one major (~9.4 kb) and two minor (~7.0 and ~5.0 kb) IR transcripts, consistent with the molecular sizes of multiple species of IR mRNAs; these multiple transcripts encompass, in addition to the coding regions, different lengths of 5′- and 3′-untranslated regions (Yamamoto et al., 2000; Saitoh et al., 2002). IR mRNA levels were normalized against GAPDH mRNA levels (Fig. 4B). Levels of 9.4-, 7.0-, and 5.0-kb IR mRNAs were decreased, respectively, by 23, 19, and 20% as soon as 6 h after acetoacetate (10 mM) treatment and remained decreased at 12 and 24 h.

The steady-state level of mRNA is dependent on gene transcription, processing of heterogeneous nuclear RNA to mRNA, and mRNA stability. Because mRNA stability is the major determinant in the control of gene expression (Guhaniyogi and Brewer, 2001), we measured the degradation rate of IR mRNAs by using actinomycin D, an inhibitor of RNA synthesis. Figure 4C shows that cells were treated with or without 10 mM acetoacetate for the first 6 h, then exposed to actinomycin D in the continuous absence or presence of acetoacetate, and subjected to Northern blot analysis at the indicated times. The level of IR mRNA (~9.4 kb) decreased more rapidly between 4 and 8 h in

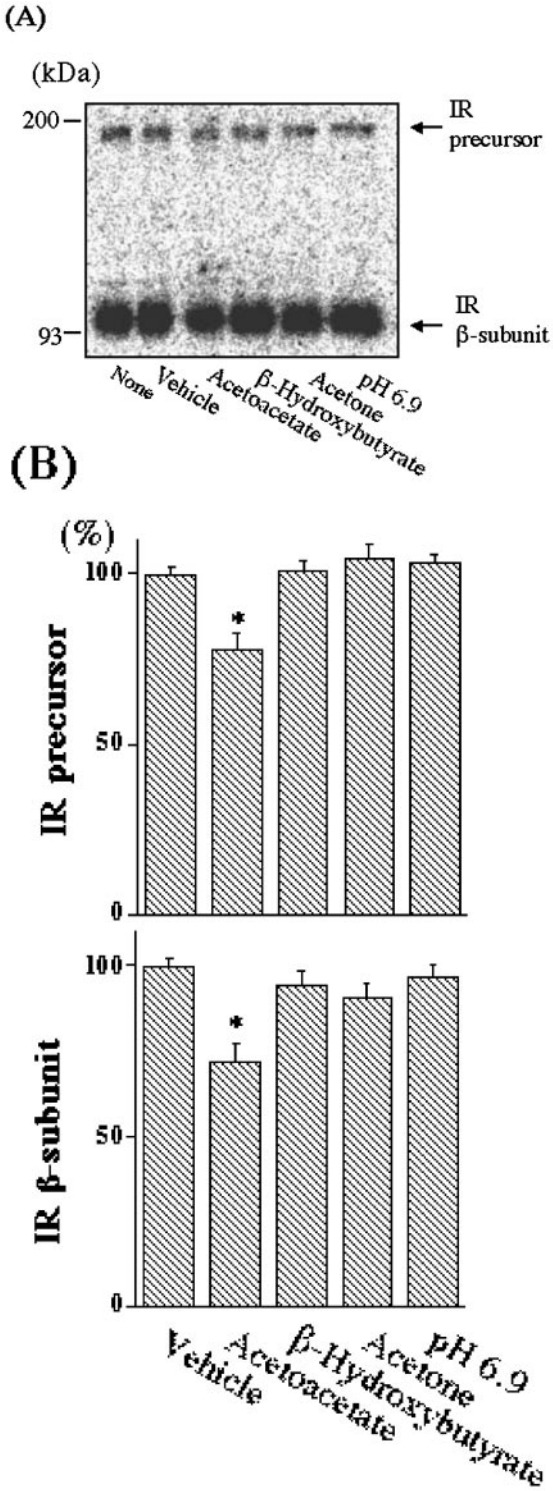

Fig. 3. Western blot analysis of the IR β-subunit and IR precursor levels: reduction by chronic treatment with acetoacetate, but not with β-hydroxybutyrate, acetone, and acidic medium. A, cells were treated without (none) or with DMSO (vehicle), 10 mM acetoacetate, 10 mM β-hydroxybutyrate, 100 mM acetone or acidic medium (pH 6.9) for 24 h and solubilized in SDS electrophoresis sample buffer; proteins were separated by SDS-PAGE under the reducing condition, transferred to membrane, and then subjected to immunoblot analysis using antibody raised against IR β-subunit. Blot shown is typical from three independent experiments with similar results. B, levels of IR β-subunit and IR precursor in A were quantified by a Bioimage analyzer; the level obtained in nontreated cells (none) is assigned a value of 100%. *, P < 0.05 compared with DMSO-treated cells.

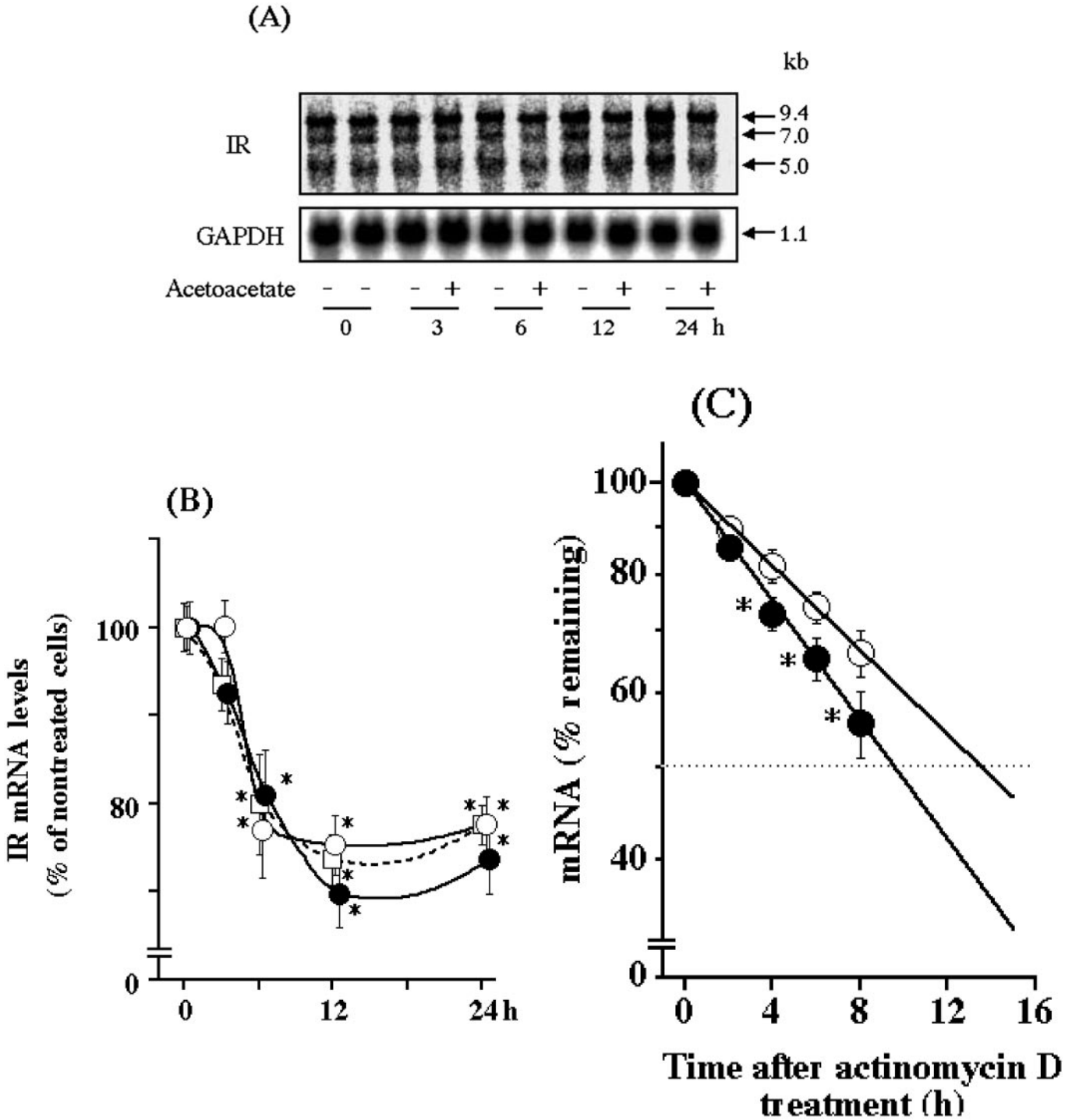

Fig. 4. Northern blot analysis of IR mRNA levels: reduction by chronic treatment with acetoacetate. A, cells were treated with (+) or without (−) 10 mM acetoacetate for up to 24 h; poly(A)$^+$RNA was extracted, electrophoresed on agarose gel, and transferred to membrane. The membrane was sequentially hybridized to each ^{32}P-labeled cDNA probe for IR and GAPDH after removing the former probe. Data are typical from three separate experiments with similar results. B, levels of IR mRNAs and GAPDH mRNA in A were quantified by a Bioimage analyzer, and the relative amounts of IR mRNAs [9.4 kb (○), 7.0 kb (●), and 5.0 kb (□)]/GAPDH mRNA were calculated. A value of 100% represents the relative level obtained in nontreated cells at the left lane in each incubation time. C, cells were pretreated for 6 h with or without 10 mM acetoacetate (Fig. 4B) and incubated with 10 μg/ml actinomycin D in the continuous absence or presence of acetoacetate. At the indicated times, poly(A)$^+$RNA was isolated and subjected to Northern blot analysis. The level of IR major mRNA (~9.4 kb) was quantified by a Bioimage analyzer. Mean ± S.E.M. (n = 3). *, $P < 0.05$ compared with acetoacetate-nontreated cells.

acetoacetate-treated cells compared with nontreated cells; the calculated half-life ($t_{1/2}$) of IR mRNA was shortened by acetoacetate from 13.6 to 9.5 h.

Attenuation of Insulin-Induced Tyrosine-Phosphorylation of IRS-1 in Acetoacetate-Treated Cells: No Effect on IRS-1 Level. Because IRS-1 mediates most, if not all,

effects of IR (Cheatham and Kahn, 1995; Aspinwall et al., 2000), we examined whether acetoacetate-induced down-regulation of cell surface IR may decrease the intrinsic tyrosine kinase activity of IR, thereby affecting insulin-induced tyrosine-phosphorylation of IRS-1. In nontreated and acetoacetate-treated (10 mM for 24 h) cells (Fig. 5A), the cell lysates were immunoprecipitated with IRS-1 antibody, followed by SDS-PAGE, and then subjected to immunoblot analysis with IRS-1 antibody; cellular levels of IRS-1 were comparable between nontreated and acetoacetate-treated cells (upper panel, lanes 1 to 4). In contrast, when nontreated and acetoacetate-treated cells were incubated with or without 100 nM insulin for 10 min (lower panel, lanes 1 to 4), insulin-induced tyrosine-phosphorylation of IRS-1 was attenuated by 56% in acetoacetate-treated cells compared with nontreated cells (Fig. 5B).

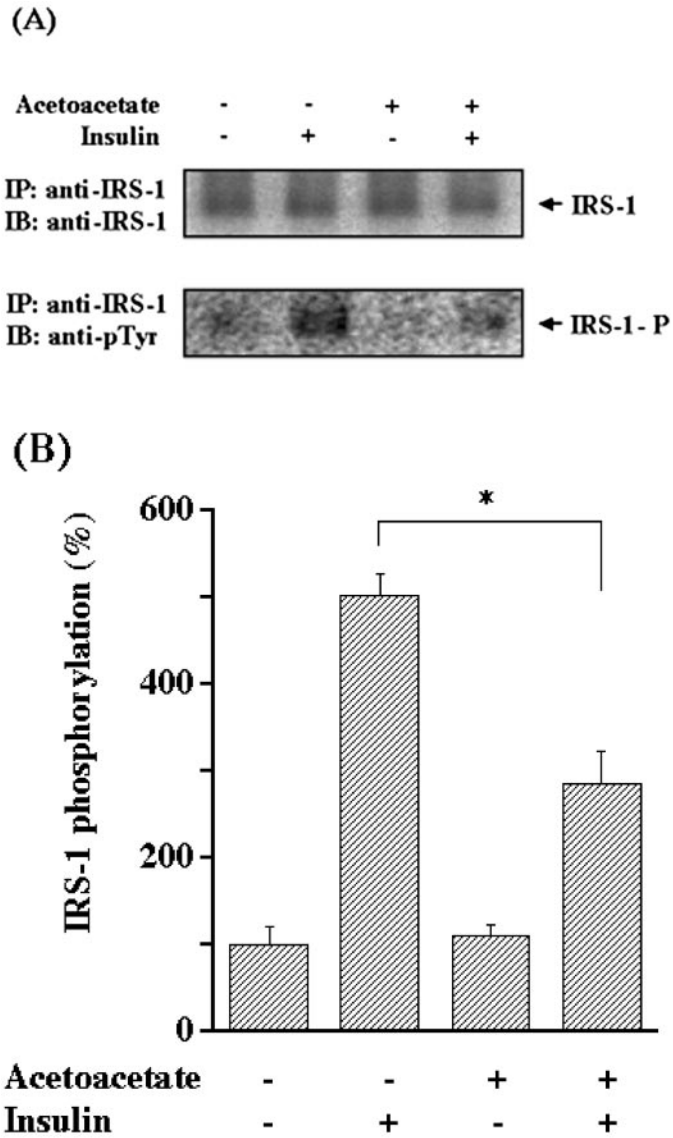

Fig. 5. Reduction of insulin-induced tyrosine-phosphorylation of IRS-1 in acetoacetate-treated cells. A, cells were treated with (+) or without (−) 10 mM acetoacetate for 24 h and incubated with (+) or without (−) 100 nM insulin for 10 min. Cell lysates were subjected to immunoprecipitation (IP) with IRS-1 antibody; the immunoprecipitates were separated by SDS-PAGE, transferred to membrane, and subjected to immunoblot (IB) analysis using IRS-1 antibody (upper panel) or phosphotyrosine-specific antibody (anti-pTyr) (lower panel). Data are typical from three independent experiments with similar results. IRS-1-P, tyrosine-phosphorylated form of IRS-1. B, levels of insulin-induced tyrosine-phosphorylation of IRS-1 in A were quantified by a Bioimage analyzer; a value of 100% represents the basal phosphorylation in cells that were incubated at 37°C for 24 h in the absence of acetoacetate and DMSO. Mean ± S.E.M. (n = 3). *, $P < 0.05$ compared with nontreated cells.

Discussion

Down-Regulation of Cell Surface IR Caused by Chronic Treatment with Acetoacetate. Our present study showed that chronic (≧24 h) treatment with acetoacetate (≧3 mM) decreased ^{125}I-insulin binding capacity and cellular levels of the IR precursor molecule and mature IR β-subunit, which were attributed to the retardation of IR synthesis. Northern blot analysis documented that acetoacetate treatment lowered IR mRNA levels by ~23% as early as 6 h when cell surface ^{125}I-insulin binding capacity was not yet decreased in acetoacetate-treated cells. The decrease of IR mRNA levels further developed at 12 and 24 h, and reduction of cell surface ^{125}I-insulin binding became evident between 12 and 24 h. In addition, acetoacetate shortened $t_{1/2}$ of IR mRNA from 13.6 to 9.5 h. Thus, acetoacetate may retard IR synthesis by lowering steady-state levels of IR mRNAs, which is attributed, at least in part, to the decreased stability of IR mRNA in acetoacetate-treated cells. mRNA stability is regulated by the specific interaction between nucleotide *cis*-elements and *trans*-acting nucleotide regulatory proteins; the *trans*-acting proteins are constitutively expressed or stimuli-inducible and shuttle between nucleus and cytoplasm (Shyu and Wilkinson, 2000; Guhaniyogi and Brewer, 2001). Nucleotide *cis*-elements and *trans*-acting nucleotide regulatory proteins that regulate transcription of the IR gene have been increasingly identified (Yoshizato et al., 2001). Much, however, remains unknown about the extra- and intracellular signaling molecules that regulate IR mRNA stability. Our present study may provide the first evidence that chronic treatment with acetoacetate down-regulates IR mRNA levels by destabilizing IR mRNA, thus lowering synthesis and cell surface expression of IR. In addition, the reduction of ^{125}I-insulin binding caused by acetoacetate was reversible as early as 12 h after the washout of acetoacetate-treated (10 mM for 24 h) cells. This observation raises the possibility that acetoacetate may regulate the stability of IR mRNA in a moment-to-moment manner.

Biological Relevance of Acetoacetate-Induced Down-Regulation of Cell Surface Functional IR. In acetoacetate-treated (10 mM for 24 h) cells, our present study showed that insulin-induced tyrosine-phosphorylation of IRS-1 was attenuated by 56%, with no change in the level of IRS-1. Because the K_d values of cell surface ^{125}I-insulin binding were similar between nontreated and acetoacetate-treated cells, the attenuation of insulin-induced tyrosine-phosphorylation of IRS-1 in acetoacetate-treated cells is attributed to the acetoacetate-induced down-regulation of cell surface functional IR. In contrast to our present study, Dikic et al. (1994) documented that increased expression of IR in PC12 cells caused enhancement of insulin-induced activation of IRS-1, Sos, and Shc, as well as nuclear translocation of mitogen-activated protein kinase that normally remained in the cytoplasm following insulin stimulation in the native PC12 cells. Also, the increased expression of IR in PC12 cells converted insulin's biological effect from cell proliferation to cell differentiation into the neuronal cells (Dikic et al., 1994). Thus, acetoacetate-induced aberrant down-regulation of cell surface IR and the subsequent attenuated tyrosine-

phosphorylation of IRS-1 may perturb insulin's biological effects in a quantitative and qualitative manner.

In peripheral efferent and afferent myelinated neurons, Sugimoto et al. (2000) documented that IR are localized at the node of Ranvier, where Na^+ channels are concentrated. This finding is reminiscent of our previous study in adrenal chromaffin cells in which activation of IR by insulin up-regulated cell surface expression of functional Na^+ channels (Yamamoto et al., 1996). In experimental animal and human insulin-dependent diabetes mellitus, insulin deficiency culminated in the disruption of paranodal myelination, independent of hyperglycemia, and the subsequent abnormal dislocation of Na^+ channels from nodal to paranodal axolemma caused down-regulation of nodal Na^+ channels and defective nerve conduction. These structural abnormalities are characteristic of diabetic neuropathy in insulin-dependent diabetes mellitus (but not in noninsulin-dependent diabetes mellitus) (Cherian et al., 1996; Sugimoto et al., 2000). In streptozotocin-induced diabetic rats, conduction velocity of peripheral motor and sensory neurons became defective; the defect, however, was totally prevented only at the neuronal site receiving local injection of low dose of insulin (Singhal et al., 1997).

Ketone bodies can freely diffuse across the cell membrane and provide the majority of brain's energy requirement during prolonged fasting (Laffel, 1999). In the brain, IR are widely distributed at much higher level in neurons than in glial cells (Zhao and Alkon, 2001) and regulate multiple processes such as cell survival, synaptogenesis, neurite extension (de Pablo and de la Rosa, 1995; Gispen and Biessels, 2000; Zhao and Alkon, 2001), and food intake/body weight (Brüning et al., 2000; Obici et al., 2002), as well as even the glucose metabolism in liver (Obici et al., 2002). Also, evidence has accumulated that brain IR promote spermatogenesis and ovarian follicle maturation via pituitary luteinizing hormone (Brüning et al., 2000), while increasing efficiency of synaptic transmission, learning, and memory (Zhao et al., 1999; Gispen and Biessels, 2000; Zhao and Alkon, 2001). IR and IR mRNA levels were significantly lower in substantia nigra of Parkinson's disease brain compared with normal and other neurodegenerative disease brains (Moroo et al., 1994; Takahashi et al., 1996). In Alzheimer's disease brain, insulin concentration was lower, and IR signaling was impaired compared with age-matched healthy humans (Zhao and Alkon, 2001; Hoyer, 2002). Insulin is synthesized by neurons in the brain and is also transported to the brain from plasma via the cerebrospinal fluid (de Pablo and de la Rosa, 1995; Gispen and Biessels, 2000; Zhao and Alkon, 2001). Intravenous infusion of insulin caused marked memory facilitation in patients with Alzheimer's disease, independent of glucose metabolism (Craft et al., 1996). Abnormal hyperphosphorylation of tau, a neuronal microtubule-associated protein, is responsible for the pathogenetic neurofibrillary lesions of Alzheimer's disease (Gasparini et al., 2002), whereas insulin reduced tau phosphorylation by inhibiting glycogen-synthase kinase-3 in cultured human neurons (Hong and Lee, 1997). Also, insulin regulates synthesis, secretion, and degradation of β-amyloid protein, which forms the pathogenetic senile plaque in Alzheimer's disease brain (Gasparini et al., 2001; 2002). In human placental plasma membranes, Xie et al. (2002) recently demonstrated that β-amyloid protein competitively inhibited ^{125}I-insulin binding to IR and attenuated insulin-induced autophosphorylation of IR. Thus, defective IR signaling has received widespread attention as the pathogenetic factor for Parkinson's disease (Sandyk, 1993) and Alzheimer's disease (Gasparini et al., 2002; Hoyer, 2002). Because ketogenic diet has been successfully employed to treat epilepsy and is proposed for the treatment of various neurodegenerative diseases, including Alzheimer's and Parkinson's diseases (Veech et al., 2001), it is essential to examine whether ketone bodies could change cell surface expression of IR and IR signaling in various peripheral and central tissues.

Acknowledgments

We thank Drs. Graeme Bell and Donald F. Steiner, as well as Sankyo Co. Ltd., for donating the plasmid pSELECT HIR and troglitazone, respectively. The technical and secretarial assistance by Keiko Kawabata and Keizo Masumoto is greatly appreciated.

References

Aspinwall CA, Qian W-J, Roper MG, Kulkarni RN, Kahn CR, and Kennedy RT (2000) Roles of insulin receptor substrate-1, phosphatidylinositol 3-kinase and release of intracellular Ca^{2+} stores in insulin-stimulated insulin secretion in β-cells. *J Biol Chem* **275:**22331–22338.

Boden G, Lebed B, Schatz M, Homko C, and Lemieux S (2001) Effects of acute changes of plasma free fatty acids on intramyocellular fat content and insulin resistance in healthy subjects. *Diabetes* **50:**1612–1617.

Brüning JC, Gautam D, Burks DJ, Gillette J, Schubert M, Orban PC, Klein R, Krone W, Muller-Wieland D, and Kahn CR (2000) Role of brain insulin receptor in control of body weight and reproduction. *Science (Wash DC)* **289:**2122–2125.

Cheatham B and Kahn CR (1995) Insulin action and the insulin signaling network. *Endocr Rev* **16:**117–142.

Cherian PV, Kamijo M, Angelides KJ, and Sima AAF (1996) Nodal Na^+-channel displacement is associated with nerve-conduction slowing in the chronically diabetic BB/W rat: prevention by aldose reductase inhibition. *J Diabetes Complicat* **10:**192–200.

Craft S, Newcomer J, Kanne S, Dagogo-Jack S, Cryer P, Sheline Y, Luby J, Dagogo-Jack A, and Alderson A (1996) Memory improvement following induced hyperinsulinemia in Alzheimer's disease. *Neurobiol Aging* **17:**123–130.

de Pablo F and de la Rosa EJ (1995) The developing CNS: a scenario for the action of proinsulin, insulin and insulin-like growth factors. *Trends Neurosci* **18:**143–150.

Dikic I, Schlessinger J, and Lax I (1994) PC12 cells overexpressing the insulin receptor undergo insulin-dependent neuronal differentiation. *Curr Biol* **4:**702–708.

Féry F and Balasse EO (1985) Ketone body production and disposal in diabetic ketosis. A comparison with fasting ketosis. *Diabetes* **34:**326–332.

Gasparini L, Gouras GK, Wang R, Gross RS, Beal MF, Greengard P, and Xu H (2001) Stimulation of β-amyloid precursor protein trafficking by insulin reduces intraneuronal β-amyloid and requires mitogen-activated protein kinase signaling. *J Neurosci* **21:**2561–2570.

Gasparini L, Hetzer TJ, Greengard P, and Xu H (2002) Does insulin dysfunction play a role in Alzheimer's disease? *Trends Pharmacol Sci* **23:**288–293.

Gispen WH and Biessels G-J (2000) Cognition and synaptic plasticity in diabetes mellitus. *Trends Neurosci* **23:**542–549.

Guhaniyogi J and Brewer G (2001) Regulation of mRNA stability in mammalian cells. *Gene* **265:**11–23.

Hashimoto T, Cook WS, Qi C, Yeldandi AV, Reddy JK, and Rao MS (2000) Defect in peroxisome proliferator-activated receptor α-inducible fatty acid oxidation determines the severity of hepatic stenosis in response to fasting. *J Biol Chem* **275:** 28918–28928.

Hong M and Lee VM-Y (1997) Insulin and insulin-like growth factor-1 regulate tau phosphorylation in cultured human neurons. *J Biol Chem* **272:**19547–19553.

Hoyer S (2002) The brain insulin signal transduction system and sporadic (type II) Alzheimer disease: an update. *J Neural Transm* **109:**341–360.

Jackerott M, Baudry A, Lamothe B, Bucchini D, Jami J, and Joshi RL (2001) Endocrine pancreas in insulin receptor-deficient mouse pups. *Diabetes* **50** (**Suppl 1):**S146–S149.

Laffel L (1999) Ketone bodies: a review of physiology, pathophysiology and application of monitoring to diabetes. *Diabetes Metab Res Rev* **15:**412–426.

Leibiger IB, Leibiger B, Moede T, and Berggren PO (1998) Exocytosis of insulin promotes insulin gene transcription via the insulin receptor/PI-3 kinase/p70 S6 kinase and CaM kinase pathways. *Mol Cell* **1:**933–938.

Moroo I, Yamada T, Makino H, Tooyama I, McGeer PL, McGeer EG, and Hirayama K (1994) Loss of insulin receptor immunoreactivity from the substantia nigra pars compacta neurons in Parkinson's disease. *Acta Neuropathol* **87:**343–348.

Obici S, Feng Z, Karkanias G, Baskin DG, and Rossetti L (2002) Decreasing hypothalamic insulin receptors causes hyperphagia and insulin resistance in rats. *Nat Neurosci* **5:**556–572.

Patti M-E (1999) Nutrient modulation of cellular insulin action. *Ann NY Acad Sci* **892:**187–203.

Saitoh T, Yanagita T, Shiraishi S, Yokoo H, Kobayashi H, Minami S, Onitsuka T, and Wada A (2002) Down-regulation of cell surface insulin receptor and insulin receptor substrate-1 phosphorylation by inhibitor of 90-kDa heat-shock protein family: endoplasmic reticulum retention of monomeric insulin receptor precursor with calnexin in adrenal chromaffin cells. *Mol Pharmacol* **62:**847–855.

Sandyk R (1993) The relationship between diabetes mellitus and Parkinson's disease. *Int J Neurosci* **69:**125–130.

Shiraishi S, Yamamoto R, Yanagita T, Yokoo H, Kobayashi H, Uezono Y, and Wada A (2001) Down-regulation of cell surface insulin receptors by sarco(endo)plasmic reticulum Ca^{2+}-ATPase inhibitor in adrenal chromaffin cells. *Brain Res* **898:**152–157.

Shiraishi S, Yokoo H, Kobayashi H, Yanagita T, Uezono Y, Minami S, Takasaki M, and Wada A (2000) Post-translational reduction of cell surface expression of insulin receptors by cyclosporin A, FK506 and rapamycin in bovine adrenal chromaffin cells. *Neurosci Lett* **293:**211–215.

Shyu A-B and Wilkinson MF (2000) The double lives of shuttling mRNA binding proteins. *Cell* **102:**135–138.

Singhal A, Cheg C, Sun H, and Zochodone DW (1997) Near nerve local insulin prevents conduction slowing in experimental diabetes. *Brain Res* **763:**209–214.

Sugimoto K, Murakawa Y, Zhang W, Xu G, and Sima AAF (2000) Insulin receptor in

rat peripheral nerve: its localization and alternatively spliced isoforms. *Diabetes Metab Res Rev* **16:**354–363.

Takahashi M, Yamada T, Tooyama I, Moroo I, Kimura H, Yamamoto T, and Okada H (1996) Insulin receptor mRNA in the substantia nigra in Parkinson's disease. *Neurosci Lett* **204:**201–204.

Veech RL, Chance B, Kashiwaya Y, Lardy HA, and Cahill GF Jr (2001) Ketone bodies, potential therapeutic uses. *IUBMB Life* **51:**241–247.

Wilson SP, Viveros OH, and Kirshner N (1985) Relationship between the regulation of enkephalin-containing peptide and dopamine-β-hydroxylase levels in cultured adrenal chromaffin cells. *J Neurochem* **45:**1363–1370.

Willson TM, Lambert MH, and Kliewer SA (2001) Peroxisome proliferator-activated receptor γ and metabolic disease. *Annu Rev Biochem* **70:**341–367.

Xie L, Helmerhorst E, Taddei K, Plewright B, van Bronswijk W, and Martins R (2002) Alzheimer's β-amyloid peptides compete for insulin binding to the insulin receptor. *J Neurosci* **22:**RC221(1–5).

Xu J and Tse FW (1999) Brefeldin A increases the quantal size and alters the kinetics of catecholamine release from rat adrenal chromaffin cells. *J Biol Chem* **274:**19095–19102.

Yamamoto R, Kobayashi H, Yanagita T, Yokoo H, Kurose T, Shiraishi S, Minami S, Matsukura S, and Wada A (2000) Up-regulation of cell surface insulin receptor by protein kinase C-α in adrenal chromaffin cells: involvement of transcriptional and translational events. *J Neurochem* **75:**672–682.

Yamamoto R, Yanagita T, Kobayashi H, Yuhi T, Yokoo H, and Wada A (1996) Up-regulation of functional voltage-dependent sodium channels by insulin in cultured bovine adrenal chromaffin cells. *J Neurochem* **67:**1401–1408.

Yoshizato K, Shirotani T, Furukawa N, Taguchi T, Motoshima H, Toyonaga T, Hirashima Y, Kawashima J, Ebina Y, Shichiri M, and Araki E (2001) Identification of a *cis*-acting element and a novel *trans*-acting factor of the human insulin receptor gene in HepG2 and rat liver cells. *Biochem Biophys Res Commun* **280:**428–434.

Zhao W, Chen H, Xu H, Moore E, Meiri N, Quon MJ, and Alkon DL (1999) Brain insulin receptors and spatial memory. Correlated changes in gene expression, tyrosine phosphorylation and signaling molecules in the hippocampus of water maze trained rats. *J Biol Chem* **274:**34893–34902.

Zhao W-Q and Alkon DL (2001) Role of insulin and insulin receptor in learning and memory. *Mol Cell Endocrinol* **177:**125–134.

Address correspondence to: Akihiko Wada, Department of Pharmacology, Miyazaki Medical College, Kiyotake, Miyazaki 889-1692, Japan. E-mail: akihiko@fc.miyazaki-med.ac.jp