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

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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 125I-insulin binding by ~38%, with no change in the Kd value. The reduction of 125I-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 IRS-1 synthesis, thereby retarding insulin-induced activation of IRS-1.

Phyisological 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, intra-chain disulfide-bond formation/isomerization, and disulfide-linked homodimerization at the endoplasmic reticulum (ER). The homodimeric IR precursor is proteolytically processed into the disulfide-linked ααββ complex at the trans-Golgi network, which is transported to the plasma membrane via an

ABBREVIATIONS: FFA, free fatty acids; IR, insulin receptors; ER, endoplasmic reticulum; IRS-1, insulin receptor substrate-1; cPGL2, 6α-carboxyprostaglandin I2; 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 Ca²⁺ 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, peptideyl prolyl cis-trans-isomerase activity of immunophlinins (Shiraishi et al., 2000) and Ca²⁺-ATPase activity of the ER (Shiraishi et al., 2001) promoted cell surface externalization of IR from the trans-Golgi network. In the present study, 125I-insulin binding, immunoblot, and Northern blot analyses show that chronic treatment with acetocetate (but not β-hydroxybutyrate, acetone, and acetic 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 NaN₃ were from Nacalai Tesque (Kyoto, Japan). Acetocetate, d(-)-β-hydroxybutyrate, clotrbibrate, 6α-carba-prostaglandin I₂ (cPGI), oleic acid, arachidonic acid, eicosapentaenoic acid, brefeldin A, and actinomycin D were from Sigma-Aldrich (St. Louis, MO). ATE was from Wako Pure Chemicals (Osaka, Japan). Troglozitation 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 Oligo-3T3<sup>Super+</sup> 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). 125I-Labeled donkey anti-rabbit IgG, 125I-insulin (< 2000 Ci/mmol), and [a-32P]dCTP (> 4000 Ci/mmol) were from Amersham Bioscience (Piscataway, NJ). 125I-Insulin was diluted with nonradioactive human insulin Humulin R (Eli Lilly, Kobe, Japan), and 125I-insulin (3.125 Ci/ml) was used for the 125I-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 x 10⁶ per dish, Falcon; 35-mm diameter) in Eagle’s minimum essential medium containing 10% calf serum under 5% CO₂/95% air in a CO₂ incubator (Yamamoto et al., 1996, 2000). Three days (60–62 h) later, the cells were treated in the fresh culture medium with or without acetocetate, β-hydroxybutyrate, acetone, clotrbibrate, cPGI₂ 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 125I-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), 125I-insulin binding was similar between purified and conventional chromaffin cells. Also, acetocetate treatment (10 mM for 24 h) decreased 125I-insulin binding by 27 and 28% in purified and conventional chromaffin cells compared with nontreated cells in each cell group.

125I-Insulin Binding. Cells were washed with ice-cold Krebs-Ringer phosphate (KRP) buffer (154 mM NaCl, 5.6 mM KCl, 1.1 mM MgSO₄, 2.2 mM CaCl₂, 0.85 mM Na₂HPO₄, 2.15 mM Na₃PO₄, 5 mM glucose, and 0.5% bovine serum albumin, pH 7.4) and incubated with 0.025 to 10 nM 125I-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<sub>max</sub> and K<sub>D</sub> values of 125I-insulin binding correspond to the binding of 125I-insulin to IR (but not insulin-like growth factor receptor), as reported previously (Yamamoto et al., 1996, 2000; Shiraishi et al., 2000, 2001). 125I-Insulin binding represents cell surface (but not internalized) IR because 125I-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 Ca²⁺-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% 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-5% polyacrylamide gel electrophoresis (PAGE), transferred onto polyvinylidene difluoride (PVDF) membranes, and probed with an IR polyclonal antibody (1:1000). The bands were labeled with 125I-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 guanidinium-thiocyanate-phenolchloroform extraction using TRIzol reagent. poly(A)⁺ RNA was purified by Oligotex-dT30<sup>Super+</sup>, 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 onto a nylon membrane (Hybond-N; Amersham Biosciences) in 20× saline-sodium-citrate solution (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 SalI, and GAPDH cDNA (1.1 kilobase pairs) were labeled with [a-32P]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, 0.1% 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 12,000g 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 Na3VO4 and subjected to immunoprecipitation with IRS-1 antibody.

For immunoblot analysis, the membrane was preincubated with Tween-20-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% NaN3 and reacted overnight at 4°C with IRS-1 antibody or phosphotyrosine-specific antibody (Saitoh et al., 2002). After repeated washings with Tween-20-buffered solution, the immunoreactive bands were labeled with [125I]-anti-rabbit IgG and analyzed by a Bioimage BAS 2000 analyzer.

**Statistical Methods.** [125I]-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 [125I]-Insulin Binding by Chronic Treatment with Acetacetate: No Effect of β-Hydroxybutyrate, Acetone, Acidic Medium, Clofibrate, cPGI2, and Troglitazone.** It has been shown that in patients with noninsulin-dependent diabetes mellitus under good nutritional status, plasma concentrations of acetacetate 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 (acetacetate plus β-hydroxybutyrate) are >1 mM in hyperketonemia and ≥3 mM in ketoadiposis, developing to ≥25 mM with the preferential increase of β-hydroxybutyrate in diabetic ketoadiposis (Laffel, 1999). As shown in Fig. 1A, treatment of adrenal chromaffin cells with 10 mM acetacetate for 24 h decreased cell surface [125I]-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 acetacetate on [125I]-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 acetacetate for the first 24 h, then washed (Fig. 1B, arrow), and incubated in the absence of acetacetate for up to 48 h; [125I]-insulin binding gradually returned to the control nontreated level between 24 and 48 h. Figure 1C shows that acetacetate decreased [125I]-insulin binding in a concentration-dependent manner between 3 and 60 mM. Rosenthal plot analysis (Fig. 1D) revealed that acetacetate (10 mM for 24 h) decreased the Bmax values, with a statistical significance (P < 0.05) from 115.5 ± 4.2 to 83.2 ± 3.8 fmol/4 × 106 cells, without altering the Kd values (3.5 ± 0.3 nM, nontreated cells; 3.4 ± 0.3 nM, acetacetate-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 acetacetate binds to PPAR, we raised the question whether ligands for PPAR decrease [125I]-insulin binding. Treatment of adrenal chromaffin cells for 24 h with 300 µM clofibrate (PPARα ligand), 20 µM cPGI2 (PPARγ/β ligand), or 50 µM troglitazone (PPARy ligand) did not alter [125I]-insulin binding (fmol/4 × 106 cells; 1.42 ± 0.08, nontreated cells; 1.47 ± 0.10, clofibrate-treated cells; 1.48 ± 0.04, cPGI2-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 [125I]-insulin binding (authors’ unpublished observation).

**Internalization Rate of Cell Surface IR: No Effect of Chronic Treatment with Acetacetate.** By using brefeldin A, we examined whether acetacetate 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, α2C-adrenoreceptors, 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 acetacetate for up to 18 h, and cell surface [125I]-insulin binding was assayed at the indicated times. In cells treated with brefeldin A alone, [125I]-insulin binding was progressively decreased in a time-dependent manner with a t1/2 of 7.5 h, a value consistent with previous studies (Shiraishi et al., 2000, 2001). The coincident treatment with acetacetate, 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 Acetacetate, 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 Kuhn, 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.
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/H9252-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 (Ghahaniyogi 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

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**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 125I-insulin binding assay. Mean ± S.E.M. (n = 5). *, P < 0.05 compared with brefeldin A-nontreated cells.

**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.

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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).

**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 125I-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 125I-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 125I-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 125I-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 $K_t$ values of cell surface 125I-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 have biological consequences similar to those seen with insulin receptor overexpression in PC12 cells.
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, synaptic transmission, neurotoxicity (de Pablo and de la Rosa, 1995; Gispen and Biessels, 2000; Zhao and Alkon, 2001), and food intake/body weight (Brüning et al., 2002; 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 in vivo IR promote spermatogenesis and ovarian follicle maturation via pituitary luteinizing hormone (Brüning et al., 2000), while in vivo IR promote spermatogenesis and ovarian follicle maturation via pituitary luteinizing hormone (Brüning et al., 2000), while in vivo IR promote spermatogenesis and ovarian follicle maturation via pituitary luteinizing hormone (Brüning et al., 2000), while in vivo IR promote spermatogenesis and ovarian follicle maturation via pituitary luteinizing hormone (Brüning et al., 2000), while in vivo IR promote spermatogenesis and ovarian follicle maturation via pituitary luteinizing hormone (Brüning et al., 2000), while in vivo IR promote spermatogenesis and ovarian follicle maturation via pituitary luteinizing hormone (Brüning et al., 2000), while in vivo IR promote spermatogenesis and ovarian follicle maturation via pituitary luteinizing hormone (Brüning et al., 2000), while in vivo IR promote spermatogenesis and ovarian follicle maturation via pituitary luteinizing hormone (Brüning et al., 2000), while in vivo IR promote spermatogenesis and ovarian follicle maturation via pituitary luteinizing hormone (Brüning et al., 2000), while in vivo IR promote spermatogenesis and ovarian follicle maturation via pituitary luteinizing hormone (Brüning et al., 2000), while in vivo IR promote spermatogenesis and ovarian follicle maturation via pituitary luteinizing hormone (Brüning et al., 2000), while in vivo IR promote spermatogenesis and ovarian follicle maturation via pituitary luteinizing hormone (Brüning et al., 2000), while in vivo IR promote spermatogenesis and ovarian follicle maturation.


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