THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 278, No. 48, Issue of November 28, pp. 47376–47387, 2003
Printed in U.S.A.

Human Glycated Albumin Affects Glucose Metabolism in L6 Skeletal Muscle Cells by Impairing Insulin-induced Insulin Receptor Substrate (IRS) Signaling through a Protein Kinase Cα-mediated Mechanism*

Received for publication, January 31, 2003, and in revised form, September 10, 2003
Published, JBC Papers in Press, September 11, 2003, DOI 10.1074/jbc.M301088200

Claudia Miele‡§¶, Audrey Riboulet§¶‖, Maria Alessandra Maitan‡, Francesco Oriente‡, Chiara Romano‡, Pietro Formisano‡, Jean Giudicelli§, Francesco Beguinot‡, and Emmanuel Van Obberghen§‡‡**

From the ‡Dipartimento di Biologia e Patologia Cellulare e Molecolare & Centro di Endocrinologia ed Oncologia Sperimentale del Consiglio Nazionale delle Ricerche, Università di Napoli Federico II, Via S. Pansini, 5, 80131 Naples, Italy and §INSERM Unité 145, Faculté de Médecine 28, Avenue de Valombrose 06107 Nice Cedex 2, France

Nonenzymatic glycation is increased in diabetes and leads to increased levels of glycated proteins. Most studies have focused on the role of glycation products in vascular complications. Here, we have investigated the action of human glycated albumin (HGA) on insulin signaling in L6 skeletal muscle cells. Exposure of these cells to HGA inhibited insulin-stimulated glucose uptake and glycogen synthase activity by 95 and 80%, respectively. These effects were time- and dose-dependent, reaching a maximum after 12 h incubation with 0.1 mg/ml HGA. In contrast, exposure of the cells to HGA had no effect on thymidine incorporation. Further, HGA reduced insulin-stimulated serine phosphorylation of PKB and GSK3, but did not alter ERK1/2 activation. HGA did not affect either insulin receptor kinase activity or insulin-induced Shc phosphorylation on tyrosine. In contrast, insulin-dependent IRS-1 and IRS-2 tyrosine phosphorylation was severely reduced in cells preincubated with HGA for 24 h. Insulin-stimulated association of PI3K with IRS-1 and IRS-2, and PI3K activity were reduced by HGA in parallel with the changes in IRS tyrosine phosphorylation, while Grb2-IRS association was unchanged. In L6 myotubes, exposure to HGA increased PKC activity by 2-fold resulting in a similar increase in Ser/Thr phosphorylation of IRS-1 and IRS-2. These phosphorylations were blocked by the PKC inhibitor bisindolylmaleimide (BDM). BDM also blocked the action of HGA on insulin-stimulated PKB and GSK3α. Simultaneously, BDM rescued insulin-stimulation of glucose uptake and glycogen synthase activity in cells exposed to HGA. The use of antibodies specific to PKC isoforms shows that this effect appears to be mediated by activated PKCα, independent of reactive oxygen species production. In summary, in L6 skeletal muscle cells, exposure to HGA leads to insulin resistance selectively in glucose metabolism with no effect on growth-related pathways regulated by the hormone.

Insulin plays a major role in regulating metabolic pathways associated with energy storage and utilization and with cellular proliferation. Following insulin binding, insulin receptor tyrosine kinase is activated, leading to the phosphorylation of several intracellular protein substrates, including IRS-1/2/3/4 proteins and Shc[1] proteins. These initial events generate multiple signaling cascades that mediate the final cellular responses to insulin (1, 2). Insulin-activated signaling modules include the Ras/ERK (3), the PI3K/PKB (4), and the PKC pathways (5, 6).

Resistance to insulin action is a common abnormality present in major human diseases such as diabetes mellitus and obesity. Insulin resistance in diabetes is genetically determined, but its incidence is also affected by environmental conditions and by factors secondary to diabetes itself (7). These acquired and secondary factors further impair insulin action in the diabetic individual. For instance, chronic hyperglycemia *per se* promotes insulin resistance (8, 9). A number of mechanisms have been proposed to explain hyperglycemia-induced insulin resistance. These include abnormalities in the PKC signaling system (10) and activation of the NF-κB transcription factors by chronically elevated glucose concentrations (11, 12). However, the molecular mechanism(s) through which hyperglycemia exacerbates insulin resistance in diabetes have only

* The research performed in INSERM Unit 145 (Nice, France) was supported by INSERM, Région PACA, Université de Nice-Sophia Antipolis, and by grants from the European Community (N°QLG1-CT-1999-00674 "Eurodiabetesgenes" and QLK3-CT-2000-01038), and from Merck Santé (Lyon, France) and Aventis (Frankfurt, Germany). The work performed in the Dipartimento di Biologia e Patologia Cellulare e Molecolare (Naples, Italy) was supported in part by the European Community (Grant QLRT-1999-00674 "Eurodiabetesgenes" (to F. B.), grants from the Associazione Italiana per la Ricerca sul Cancro (AIRC) (to F. B. and P. F.), the Ministero dell'Università e della Ricerca Scientifica (to F. B.), and Telethon-Italy (Grant no. 0896 (to F. B.)). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¶ Both authors contributed equally to this work.

‖ Supported by a Fellowship from the Région Provence-Alpes-Côte d'Azur and Bayer-Diagnostics France.

** Recipient of a fellowship of the Federazione Italiana per la Ricerca sul Cancero (FIRC).

‡‡ To whom correspondence should be addressed: INSERM Unité 145, Faculté de Médecine, 28, Avenue de Valombrose 06107 Nice Cedex 2, France. Tel.: 33-4-93-81-54-47; Fax: 33-4-93-81-54-32; E-mail: vanobbeg@unice.fr.

[1] The abbreviations used are: Shc, Src-Homology-Collagen; AGEs, advanced glycation end products; BDM, bisindolylmaleimide; CM-DCF, chloro-methyl-2′7′-dichlorofluorescein diacetate; ERK, extracellular-regulated kinase; GSK, glycogen synthase kinase; HGA, human glycated albumin; HA, human albumin (nonglycated); IRS, insulin receptor substrate; PDTC, pyrrolidonedithiocarbamate; PI3K, phosphoinositide 3-kinase; PKB, protein kinase B; PKC, protein kinase C; ROS, reactive oxygen species; UDPG, uridine 5′-diphosphate-glucose; phosphoY, phosphotyrosine; 2-DG, 2-deoxy-D-glucose.

partially been elucidated. An additional deleterious effect of chronic hyperglycemia is the increased production of advanced glycation end products (AGEs). Chronic high intracellular glucose concentrations cause an increase in both intracellular and extracellular AGEs (13, 14). AGE precursors are formed by several reactions including intracellular auto-oxidation of glucose to glyoxal (15) and breakdown of glyceraldehyde-3-phosphate and dihydroxyacetone phosphate to methylglyoxal (16). AGEs are generated through the interaction of these intracellular α-dicarbonyl precursors with the amino groups of both intra- and extracellular proteins (17). Chronic hyperglycemia also leads to the production of Amadori products through the nonenzymatic glycation reactions between glucose and reactive amino groups of serum proteins. Depending on the protein turnover rate and glucose concentration, these Amadori products undergo further irreversible reactions to form AGEs. The modifications of proteins that lead to their glycation induce alterations in their biological properties as compared with their non-glycated counterparts. Several studies have shown that elevated concentrations of Amadori products such as glycated albumin are associated with diabetic atherogenesis by activating vascular smooth muscle cells (18). Glycated albumin has also been implicated in the development of diabetic retinopathy (19) by induction of vascular endothelial growth factor expression (20, 21) and the stimulation of choroidal endothelial cell proliferation (22). Finally, glycated albumin has been shown to participitate in the development of diabetic nephropathy by the induction of the cellular formation of cytokines and growth factors (23), which may themselves contribute to diabetic renal disease (24). Glycated albumin and AGEs exert their effects through specific cellular receptors found in different cell types and through the activation of several signaling pathways (25, 26). Thus, glycated albumin-receptor binding elicits a signal transduction pathway leading to the generation of oxygen free radicals. These reactive oxygen species activate the redox-sensitive transcription factor NF-κB (18), a pleiotropic regulator of many genes. Recently, Naitoh *et al.* (27) demonstrated that, in human monocytic cells, glycated albumin induces the release of TNF-α, a factor involved in insulin resistance (28). Although glycated albumin has been linked to the vascular complications of diabetes, it is presently unclear whether exposure to glycated albumin induces resistance to insulin and at which step in the insulin-signaling cascade this may occur. We hypothesized that glycated albumin may be involved in the modulation of insulin signaling and hence in the generation of insulin resistance. To investigate this hypothesis, we analyzed the action of human glycated albumin (HGA) on insulin signaling in L6 skeletal muscle cells. We demonstrated that, in these cells, exposure to human glycated albumin selectively inhibits the PI3K/PKB branch of the insulin signaling cascade, while leaving the Ras-ERK pathway and mitogenic action of the hormone unaltered. Mechanistically, HGA-mediated PI3K/PKB inhibition is dependent on a PKCα-mediated serine/threonine phosphorylation of IRS-1/2 proteins that leads to a strong decrease in insulin-regulated metabolic responses, such as glucose uptake and glycogen synthesis. Interestingly, activation of PKCα by chronic HGA treatment appears to be independent of ROS production. Thus, by deregulating intracellular insulin signaling, human glycated albumin exacerbates the insulin-resistant state.

EXPERIMENTAL PROCEDURES

General—Media and sera for tissue culture were from Invitrogen. Phospho-PKB, phospho-ERK, IRS-1, and IRS-2 antibodies were purchased from Cell Signaling Technology (Beverly MA). PI3K and phospho-GSK-3 antibodies were from Upstate Biotechnology Inc. (Lake Placid, NY). Antibodies directed against PKC isoforms were used as previously described (29). All other antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Electrophoresis and Western blot reagents were from Bio-Rad (Richmond, VA), chloro-methyl-2′7′-dichlorofluorescein diacetate was a gift from C. Maziere (Faculté de Médecine, Amiens, France). [γ-^{32}P]ATP (3,000 Ci/mmol), [^{14}C]UDPG, [^{3}H]thymidine, 2-deoxy-D-[^{3}H]glucose, [^{32}P]orthophosphate, and ECL reagents were from Amersham Biosciences. Other reagents were from Sigma.

TABLE I
Physicochemical properties of glycated human albumin

Extent of lysine modifications was determined with TNBS and arginine modifications by the 9, 10 phenanthrenequinone method as described with "Experimental Procedures." The modification ratio of HGA was expressed as a percent of nonglycated human albumin modifications used as control.

	Non-glycated human albumin	Glycated human albumin
Fluorescent AGEs	Undetectable	Undetectable
CML/mg protein	60 ng	170 ng
Lys modification (%)	100	91.9 ± 5.2
Arg modification (%)	100	98.6 ± 0.5
IGF-1	Undetectable	Undetectable
LPS	Undetectable	Undetectable

Characterization of Glycated Human Serum Albumin—Glycated and nonglycated human serum albumin were purchased from Sigma Chemical Co. The glycated serum albumin contained 2.7–3.5 mol of fructosamine per mol of albumin. The human glycated and nonglycated albumin preparations were tested for (i) fluorescent advanced glycation end products concentrations, determined by fluorescence assays (from 360 to 600 nm) upon excitation at 370 nm or 350 nm (30), (ii) CML concentrations were determined by using a CML-ELISA kit which uses carboxymethyl caproate as standards as described in (31), and (iii) the extent of lysine and arginine modifications, measured using the 2,4,6-trinitrobenzen-suffonic acid and 9,10-phenanthrenequinone methods, respectively (32, 33). Each batch was tested for possible insulin-like growth factor-I contamination by IGF-I-D-RIA-CT (BioSource Europe, Nivelles, Belgium) and the absence of endotoxin (LPS) by the use of Limulus amebocyte lysates assay (Sigma). Each human glycated albumin batch was reconstituted at 10 mg/ml with sterile PBS, and in order to avoid glycoxydation, immediately thereafter frozen at −30 °C until use (34).

Cell Culture—The L6 skeletal muscle cells were plated (6×10^3 cells/cm^2) and grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 2% (v/v) fetal bovine serum and 2 mM glutamine. Cultures were maintained at 37 °C, in a humidified atmosphere containing 5% (v/v) CO_2. Under these culture conditions, L6 myoblasts spontaneously differentiate into myotubes upon confluence. For PKC inhibition studies, cells were pretreated with 100 nM BDM for 30 min followed by combined pretreatment with HGA and BDM. Unless otherwise stated all experiments were performed with L6 cells at the myotube stage of differentiation.

Immunoblot Analysis—Cells were solubilized for 20 min at 4 °C with lysis buffer containing 50 mM HEPES, 150 mM NaCl, 10 mM EDTA, 10 mM $Na_4P_2O_7$, 2 mM sodium orthovanadate, 50 mM NaF, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 10 μg/ml leupeptin, pH 7.4, and 1% (v/v) Triton X-100 (TAT buffer). The lysates were clarified by centrifugation at 12,000 × *g* for 15 min at 4 °C, and aliquots were either directly separated by SDS-PAGE or incubated with the indicated antibodies for 18 h at 4 °C. Immune complexes were then precipitated with protein A beads, and resuspended in Laemmli buffer. Proteins were separated by SDS-PAGE and blotted on Immobilon-P membranes (Millipore Corp., Bedford, MA). Membranes were blocked for 1 h in TBS (10 mM Tris-HCl, pH 7.4, 140 mM NaCl), containing 4% (w/v) bovine serum albumin and then incubated with the indicated antibodies. Detection of blotted proteins was performed by ECL according to the manufacturer's instruction.

In Vivo Phosphorylation Experiments—Myotubes pretreated or not with HGA for 24 h were washed twice with phosphate-free DMEM and labeled for 12 h in this medium containing 500 μCi of [^{32}P]orthophosphate (1.7 mCi/ml). At the end of labeling, after three washes with ice-cold PBS, proteins were solubilized for 15 min at 4 °C in TAT buffer. The lysates were clarified by centrifugation at 12,000 × *g* for 15 min at 4 °C and subjected to immunoprecipitation with anti-IRS-1 or IRS-2 antibodies. Immunocomplexes were washed four times with solubilization buffer, boiled in Laemmli sample buffer, and separated by SDS-PAGE. For KOH treatment, the gels were incubated for 1 h at 55 °C in 1 M KOH as described (35). [^{32}P]orthophosphate-labeled IRS-1/2 frac-

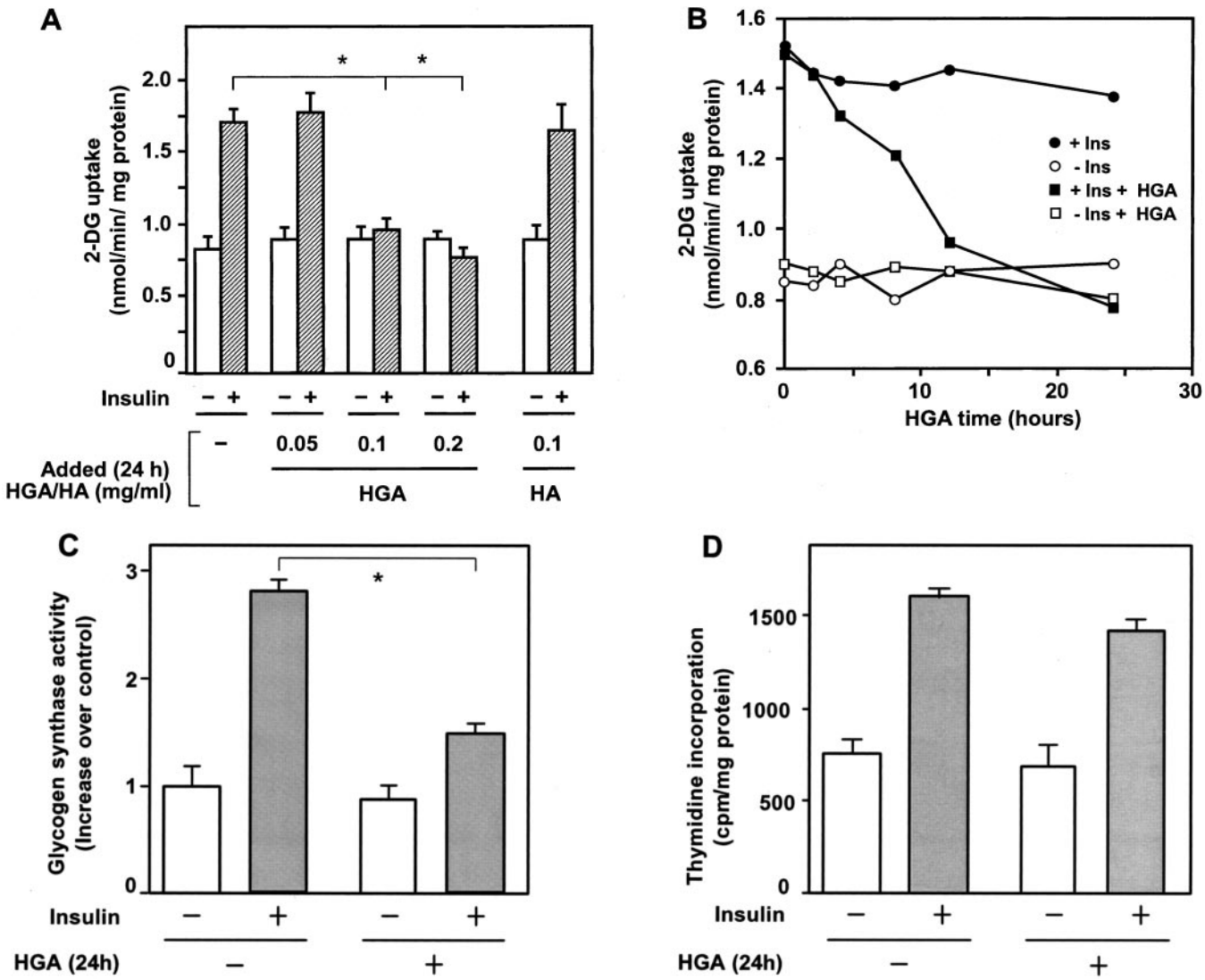

FIG. 1. **HGA action on 2-DG uptake, glycogen synthase in myotubes and thymidine incorporation in L6 myoblasts.** L6 cells were incubated with HGA or HA at different concentrations and/or different times and then stimulated with 100 nM insulin. 2-DG uptake (*A* and *B*), glycogen synthase activity (*C*), and thymidine incorporation (*D*) were then assayed as described under "Experimental Procedures." *Symbols* and *bars* represent the mean ± S.D. of triplicate determinations obtained in four (*A–C*) and three (*D*) independent experiments. *A* and *C*, *, $p < 0.001$ *versus* control insulin-stimulated group.

tions were detected with a PhosphorImager (Molecular Dynamics) and quantified (ImageQuant software).

2-Deoxy-D-glucose Uptake, Glycogen Synthase Activity, and Thymidine Incorporation Assays—These assays were performed in L6 myotubes as described in (36). Briefly, cells were incubated in serum-free α-Dulbecco's modified Eagle's medium supplemented with 0.2% (w/v) bovine serum albumin for 24 h in the presence or absence of 0.1 mg/ml HGA. Cells were incubated in glucose-free 20 mM HEPES, pH 7.4, 140 mM NaCl, 2.5 mM $MgSO_4$, 5 mM KCl, 1 mM $CaCl_2$ (HEPES buffer) and then exposed or not to 100 nM insulin for 10 min. Glucose uptake was measured by incubating cells with 20 μM 2-deoxy-D-[^{3}H]glucose (1 μCi/assay) for 10 min in HEPES buffer. The reaction was terminated by the addition of 10 μM cytochalasin B, and the cells were washed three times with ice-cold isotonic saline solution prior to lysis in 0.1 M NaOH. Incorporated radioactivity was measured in a liquid scintillation counter. Glycogen synthase activity was assayed by a modification of the described method (36). Briefly, L6 myotubes were incubated in HEPES buffer for 3 h before the assay, the cells were then stimulated with 100 nM insulin, resuspended in 10 mM EDTA and sonicated for 10 s at 300 watts. The cell suspension was centrifuged for 10 min at 2,000 × *g*, and 20-μl aliquots of the supernatants (20 μg of cell protein) were added to 60 μl of a reaction mixture containing 40 mM Tris-HCl, pH 7.8, 25 mM NaF, 20 mM EDTA, 10 mg/ml glycogen, and 7.2 mM uridine 5′-diphosphate-glucose (UDPG) and 0.05 mCi [^{14}C]UDPG in the absence or the presence of 6.7 mM glucose 6-phosphate. The supernatant reaction mixture was incubated for 20 min at 30 °C and terminated by spotting on p81 phosphocellulose filter followed by precipitation with ice-cold ethanol, and the radioactivity was counted by liquid scintillation. Enzyme activity was expressed as percent of the glucose-6-phosphate independent form (% I form).

Thymidine incorporation assays were performed as previously described (37). Briefly, cells were seeded in 6-well plates at a plating density of 10^5 cells/well. After 24 h, the culture medium was replaced with serum-free Dulbecco's modified Eagle's medium containing 0.2% (w/v) bovine serum albumin in the presence or absence of HGA, as specified for an additional 24 h. 100 nM insulin and [^{3}H]thymidine were added, and the cells were incubated for an additional 16 h.

Kinase Assays—L6 myotubes were deprived of serum for 24 h in the presence or absence of HGA and then exposed to 100 nM insulin for 10 min. For PI3K assays, after insulin stimulation, the cells were solubilized for 30 min at 4 °C in TAT buffer. After lysate clarification by centrifugation at 12,000 × *g* for 15 min at 4 °C, aliquots of the lysates were subjected to immunoprecipitation with anti-IRS-1 or anti-IRS-2 antibodies coupled to protein A-Sepharose for 2 h at 4 °C. PI3K activity was determined in IRS-1/2 immunoprecipitates as described in Filippa *et al.* (38).

For PKB assays, after insulin stimulation, the cells were solubilized for 30 min at 4 °C in TAT buffer. The lysates were clarified by centrifugation at 12,000 × *g* for 15 min at 4 °C, and PKB was immunoprecipitated using antibodies to PKB. The immune complexes were washed and assayed for PKB activity using Crosstide substrate as previously described (38). PKC activity was assayed as previously described (29).

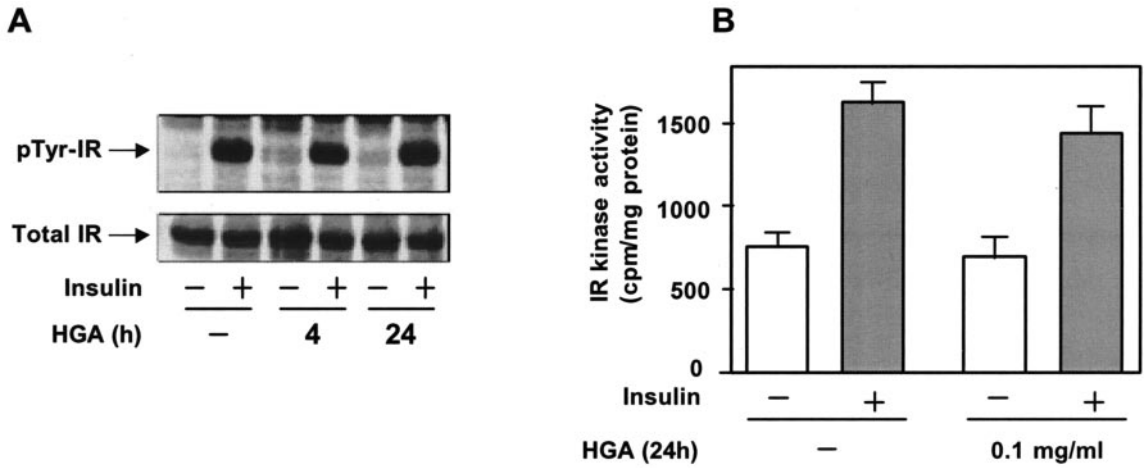

FIG. 2. **Effect of HGA on insulin receptor autophosphorylation and kinase activity.** L6 myotubes were incubated with 0.1 mg/ml HGA for the indicated times and then stimulated with 100 nM insulin (10 min). Cell lysates were immunoprecipitated with antibodies to the insulin receptor β-subunit (*A*). Precipitated proteins were separated by SDS-PAGE and blotted with antibodies either to phosphotyrosine or to insulin receptor, as indicated. Results shown are representative of three independent experiments. Alternatively, in *B*, insulin receptors were partially purified by wheat germ agglutinin-agarose chromatography from the cell lysates and *in vitro* kinase activity toward the poly(Glu-Tyr) substrate was measured as described under "Experimental Procedures." *Bars* are the mean ± S.D. of duplicate determinations of four independent experiments.

Briefly, cells were solubilized in 20 mM Tris-HCl, pH 7.5, 0.5 mM EDTA, 0.5 mM EGTA, 0.5% (v/v) Triton X-100, 25 μg/ml aprotinin, 25 μg/ml leupeptin. Cell lysates were clarified by centrifugation at 12,000 × *g* for 20 min and then supplemented with lipid activators (10 μM phorbol 12-myristate 13-acetate, 0.28 mg/ml phosphatidylserine and 4 mg/ml dioleine, final concentrations). Phosphorylation reactions were initiated by addition of 50 μM acetylated myelin basic protein (residues 4–14) substrate in 4 mM Tris, pH 7.5, 1 mM $CaCl_2$, 20 mM $MgCl_2$, 20 μM ATP, and 10 μCi of (3,000 Ci/mmol) [γ-^{32}P]ATP per ml (final concentrations). The reaction mixtures were further incubated for 10 min at room temperature, and then rapidly cooled on ice and spotted onto phosphocellulose discs. Disc-bound radioactivity was quantitated by liquid scintillation counting. PKC activity was calculated by subtracting the nonspecific kinase activity obtained in the absence of lipid activators. Activity of the specific PKC isoforms was assayed as described above, after immunoprecipitation using specific PKC isoform antibodies.

Assay for Reactive Oxygen Species Production—Intracellular production of ROS was measured by CM-DCF fluorescence as described (39). The cells were treated with H_2O_2 (500 μM) for 15 min in the absence or presence of the antioxidant PDTC (200 μM), and then exposed to 10^{-5} M CM-DCF in phosphate buffered saline for 45 min. The cells were washed three times in PBS, solubilized in H_20, and sonicated. The fluorescence was determined at 503/529 nm, normalized on a protein basis and expressed as percentages of control.

Statistical Analysis—Results are expressed as means ± S.D. Statistical significance was evaluated using the Student's *t* test for unpaired comparison. A value of $p < 0.05$ was considered statistically significant.

RESULTS

Physicochemical Properties of HGA—Table I summarizes the physicochemical properties of the human glycated albumin used in this report. We find that HGA does not contain measurable amounts of fluorescent advanced glycation end products. The CML concentrations observed in our HGA preparations are lower than the level of CML observed in diabetic plasma (32.6 ± 8.3 μg CML/ml) (40). Nagai *et al.* (41) have observed that methylglyoxal and glyoxal preferentially modified arginine rather than lysine residues. To detect the presence of modified aldehyde in human albumin, the extent of lysine and arginine modifications in human glycated and nonglycated albumin were determined. The results obtained are summarized in Table I and show the absence of significant modifications in the extent of free lysine and arginine residues between the two preparations. Lastly, the batches were found not to contain IGF-I and to be bacterial endotoxin-free. To summarize, the physicochemical properties determined for our HGA preparation demonstrate that this preparation does not contain significant AGEs products and that therefore the effects observed in L6 myotubes after 24 h of preincubation with 0.1 mg/ml of HGA are the consequence of glycated albumin present essentially as a glycated Amadori product.

Effect of HGA on Insulin Action—Insulin-resistant states are associated with alterations affecting glucose metabolism of muscle cells (42, 43). To determine whether HGA could induce insulin resistance in L6 myotubes, the effect of preincubation for 24 h with 0–0.2 mg/ml HGA on glucose transport was studied. Control cells were treated with nonglycated albumin. In control myotubes not treated with HGA or treated for 24 h with 0.1 mg/ml HA, insulin induced a 2-fold 2-DG uptake. HGA decreased insulin-stimulated glucose uptake in a dose-dependent manner without affecting basal glucose uptake. Maximal inhibition was achieved starting from 0.1 mg/ml HGA (Fig. 1*A*). To study the time course for HGA-induced insulin resistance in myotubes, cells were preincubated with 0.1 mg/ml HGA for 0–24 h. Insulin-induced glucose uptake was reduced by 60 and 75% upon HGA incubation for 4 and 8 h, respectively (decreases significant at the $p < 0.001$ level). A 12-h preincubation with 0.1 mg/ml HGA completely abolished insulin stimulation of 2-DG uptake in L6 myotubes, an inhibition conserved for at least 12 additional hours (Fig. 1*B*). Glycogen synthase activation by insulin was also inhibited by preincubation with HGA (300% increase in the absence of HGA and only 50% upon 24 h HGA preincubation, as shown in Fig. 1*C*). Similar to 2-DG uptake, the HGA-induced inhibition of glycogen synthase activation was also time- and dose-dependent, reaching a maximum after 12 h of exposure (data not shown). On the contrary, HGA pretreatment did not affect insulin-stimulated thymidine incorporation in L6 myoblasts (Fig. 1*D*).

Insulin Signaling in HGA-exposed Cells—To investigate the mechanism underlying these different actions of HGA, we focused initially on early events in insulin signaling. HGA preincubation of L6 myotubes for up to 24 h did not change insulin receptor protein levels or insulin-dependent receptor autophosphorylation on tyrosine (Fig. 2*A*). *In vitro* kinase activity of insulin receptors also remained unchanged (Fig. 2*B*). On the contrary, insulin-dependent IRS-1 and IRS-2 tyrosine phosphorylation was severely reduced in cells preincubated with HGA for 24 h (Fig. 3, *A–D*). HGA action on IRS-1 and IRS-2 phosphorylation was time-dependent with maximum inhibition

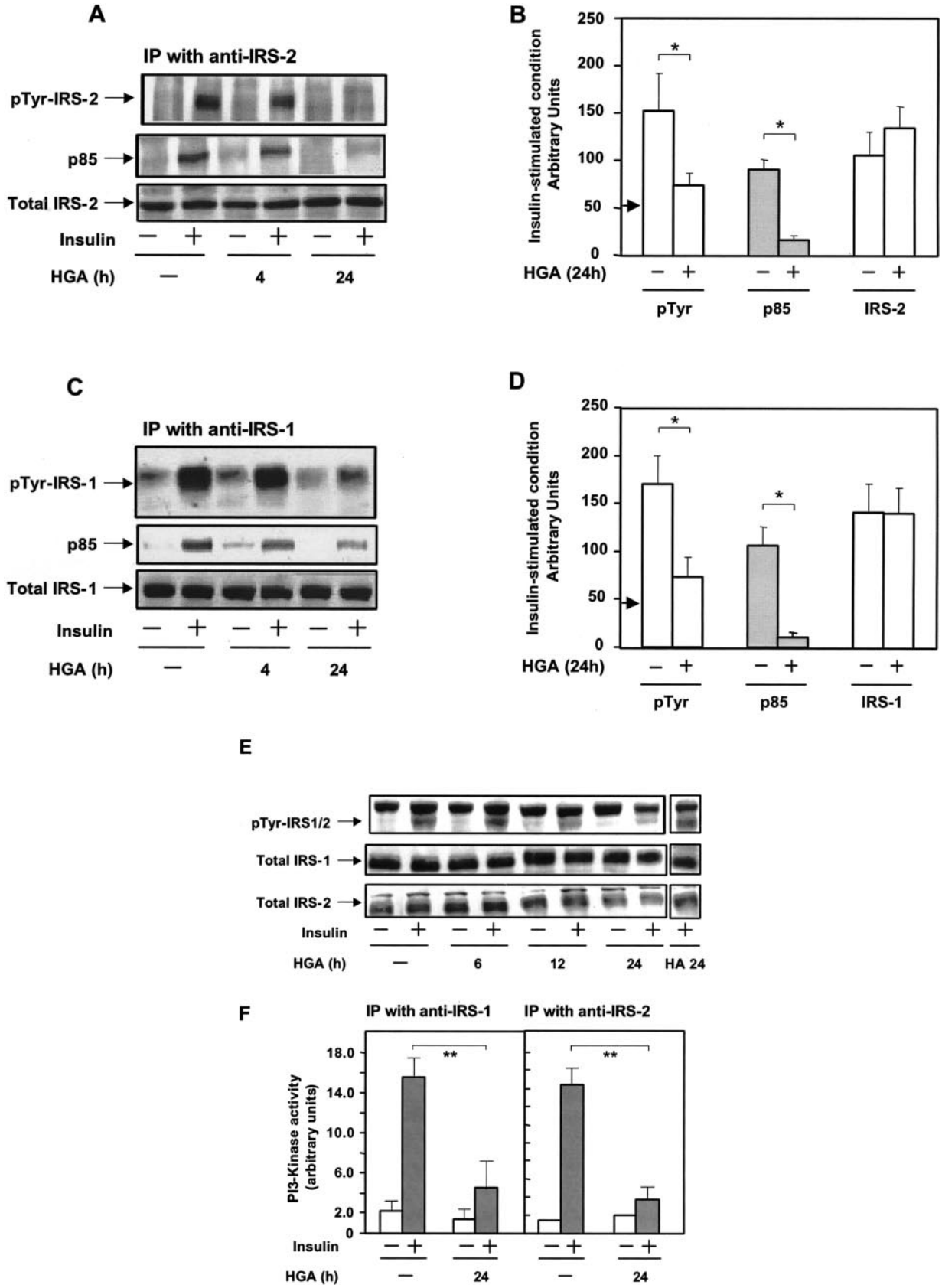

FIG. 3. **Effect of HGA on IRS-1 and IRS-2 signaling in L6 myotubes.** L6 myotubes were incubated with 0.1 mg/ml HGA or HA for the indicated times and then stimulated with 100 nM insulin (10 min). The cells were then solubilized, and the cell extracts were subjected to immunoprecipitation with antibodies to IRS-2 (*A*) or to IRS-1 (*C*), followed by blotting with antibodies to phosphotyrosine or to p85 as indicated. Aliquots of the immunoprecipitates were also subjected to blotting with antibodies to IRS-2 or to IRS-1. In *E*, cell extracts were subjected to SDS-PAGE followed by immunoblotting with antibodies to phosphotyrosine, to IRS-1 or to IRS-2. *F*, PI3K activity associated with IRS-1 and IRS-2 in basal and insulin-stimulated cells pretreated or not with 0.1 mg/ml HGA. Immunoblots of total IRS-2 (*B*) and IRS-1 (*D*) were analyzed by densitometry and means ± S.D. are shown. The experiments shown are representative of three independent experiments. An *arrow* (in *B* and *D*) indicates the basal tyrosine phosphorylation level. *B*, *D*, and *F*, *, $p < 0.05$; **, $p < 0.01$ *versus* control insulin-stimulated group.

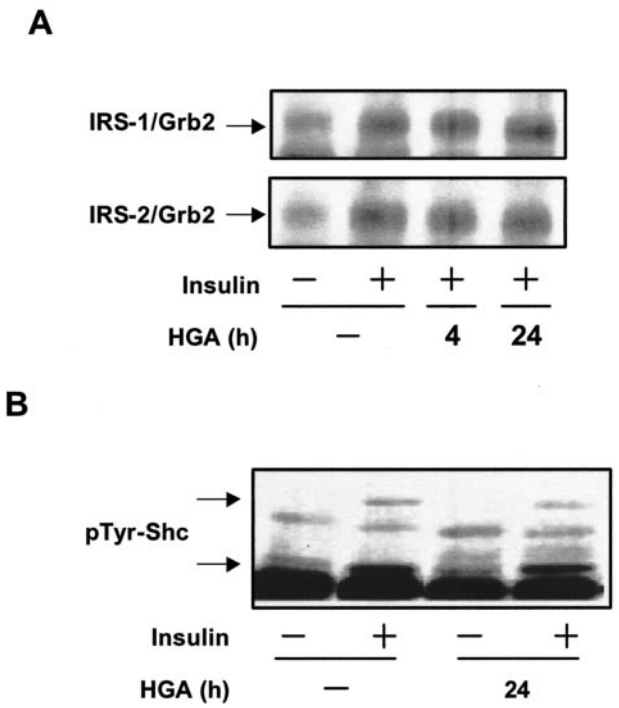

FIG. 4. **Effect of HGA on Grb2 and Shc in L6 myotubes.** L6 myotubes were incubated with 0.1 mg/ml HGA for the indicated times and thereafter stimulated with 100 nM insulin (10 min). Cell lysates were subjected to immunoprecipitation with antibodies to IRS-1 (*A*, *top panel*) or to IRS-2 (*A*, *bottom panel*) and to Shc (*B*). Immunoprecipitated proteins were separated by SDS-PAGE followed by immunoblotting with antibodies to Grb2 (*A*) or to phosphotyrosine (*B*). The experiments shown are representative of three (*A*) and four (*B*) independent experiments.

reached within 12 h of HGA exposure (Fig. 3*E*). Quantification of the total IRS-1/2 proteins indicated that the HGA-induced decrease in tyrosine phosphorylation of IRS-1/2 did not result from a reduction in these proteins as no significant differences in protein levels were observed in the HGA-treated group as compared with the untreated one (Fig. 3, *B* and *D*). No modification in the insulin-dependent tyrosine phosphorylation of IRS-1/2 was observed when L6 myotubes were pretreated with nonglycated human serum albumin (Fig. 3*E*). Western blotting of IRS-2 and IRS-1 immunoprecipitated with antibodies to p85 revealed that PI3K-IRS association decreased in parallel with IRS-2 and IRS-1 tyrosine phosphorylation in HGA-exposed cells (Fig. 3, *A–D*). Next, we determined the PI3K activity in IRS-1 and IRS-2 immune complexes. As expected, insulin induced a 7.5-fold increase in IRS-1 and IRS-2-associated PI3K activity in control cells. However, the stimulatory action of insulin on PI3K activity was virtually abolished when cells were pretreated for 24 h with HGA (Fig. 3*F*). Interestingly, IRS-Grb2 co-precipitation was not affected by exposure to HGA (Fig. 4*A*), suggesting that HGA differentially affects the tyrosine binding sites of p85 *versus* those of Grb2. Shc is also an important insulin receptor substrate with a major role in mitogenic signal transduction (1, 2). As with Grb2, HGA did not affect insulin-stimulated Shc phosphorylation in L6 myotubes (Fig. 4*B*).

To further investigate HGA action on insulin signaling, the activation state of ERK1 (p44) and ERK2 (p42), PKB and GSK3, one of the known targets of PKB, were analyzed in HGA-exposed cells. In the absence of HGA, insulin induced an increase in phosphorylation of Ser^{473} of PKB associated with a 2.5-fold increase in PKB activity (Fig. 5, *A* and *B*). HGA treatment induced a time-dependent inhibition of this PKB phosphorylation. Indeed, while Ser^{473} phosphorylation was not modified within 6 h of preincubation with HGA, it decreased to 30% of the control value within 12 h of HGA exposure ($p < 0.05$). Thereafter it remained unchanged for at least 12 additional hours (Fig. 5, *A* and *B*). In contrast, no effect of nonglycated albumin (HA) pretreatment (0.1 mg/ml) on the serine phosphorylation of PKB was observed (Fig. 5*A*). This decrease in serine phosphorylation was not accompanied by changes in the level of PKB protein expression in these cells (Fig. 5*A*). As expected, the HGA-induced block of PKB phosphorylation on Ser^{473} was associated with the disappearance of the stimulatory action of insulin on PKB activity (Fig. 5*B*). Similar results were observed when the effect of HGA on Ser-PKB phosphorylation was tested on the undifferentiated L6 myoblasts (Fig. 5*C*). GSK3, an enzyme implicated in insulin-induced glycogen synthesis, is present in two highly homologous isoforms (GSK3α and GSK3β) both of which are inhibited by PKB phosphorylation on a N-terminal serine residue (Ser^{21} in GSK3α and Ser^{9} in GSK3β). The serine phosphorylation of these two GSK3 isoforms by insulin-stimulated PKB was also inhibited by preincubation of the cells with HGA (Fig. 5*D*). As was the case for PKB phosphorylation, the effect of HGA on the phosphorylation of GSK3 was observed while the level of GSK3 protein remained unchanged. On the contrary, no effect of either HA or HGA on ERK 1 (p44) and ERK 2 (p42) expression and on insulin-stimulated phosphorylation of ERK1/2 was observed (Fig. 5*E*).

PKC Activation by HGA—In different states associated with insulin resistance, IRS serine/threonine phosphorylation decreases insulin-stimulated tyrosine phosphorylation and attenuates insulin signaling and action (44–50). To address the possibility that HGA inhibits IRS signaling through a similar mechanism, we examined the phosphorylation status of IRS-1 and IRS-2 in HGA-exposed cells. In [^{32}P]orthophosphate-labeled cells, immunoprecipitated IRS-1 and IRS-2 showed a marked basal phosphorylation (Fig. 6*A*). Quantification of the data from four individual experiments indicated that the intensity of phosphorylation increased by about 1.5- and 2.5-fold upon 24 h of exposure to HGA for IRS-1 and IRS-2, respectively (Fig. 6*B*). Alkaline treatment of the gels completely eliminated both the basal and the HGA-induced phosphorylation of IRS-1 and IRS-2 (Fig. 6*B*) indicating that this phosphorylation occurs mainly on serine/threonine residues rather than on tyrosine residues. Interestingly, as shown in Fig. 6, the HGA-induced IRS serine/threonine phosphorylation was inhibited by pretreatment of the cells with bisindolylmaleimide (BDM), a general PKC inhibitor.

Therefore, we further investigated the hypothesis that PKC might mediate the HGA effect on insulin signaling and action in L6 myotubes. To this end, the effect of HGA on total PKC activity was first analyzed. As shown in Fig. 7, HGA incubation induced a time-dependent increase in PKC activity in the L6 myotubes. This increase was already significant within 4 h incubation with HGA, reached a maximum within 12 h (data not shown), and remained unchanged for at least 12 additional hours. No effect of HGA on PKC activity was detectable in BDM-preincubated cells. Preincubation with BDM also caused complete rescue of insulin action on IRS-1 and IRS-2 phosphorylation in cells exposed to HGA for 24 h (Fig. 8, *A* and *B*). Similarly, BDM prevented the HGA-induced blockade of PKB and GSK3α serine phosphorylation by insulin (Fig. 8, *C* and *D*). It should be noted that the levels of expression of these proteins were not affected by the different treatments.

Next, we analyzed whether inhibition of PKC interfered with the ability of HGA to decrease glucose transport in L6 myotubes. Importantly, PKC inhibition with BDM almost completely rescued the insulin-stimulated glucose uptake in HGA-

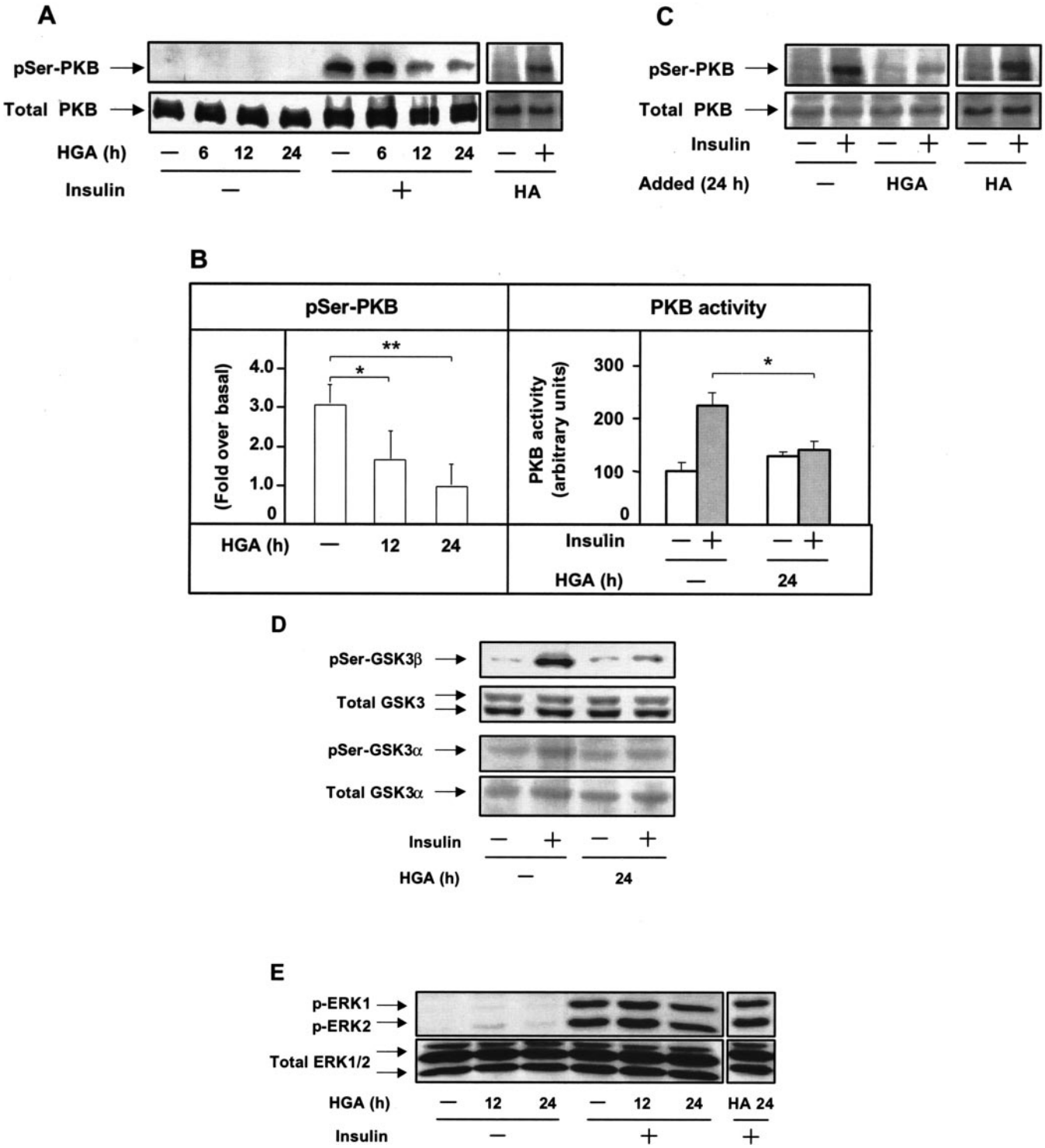

FIG. 5. **Effect of HGA on PKB, GSK-3α, GSK-3β, and ERK1/2 in L6 cells.** L6 cells were incubated with 0.1 mg/ml HGA (*A–E*) or HA (*A* and *B*) for the indicated times and thereafter stimulated with 100 nM insulin (10 min). The cells were then solubilized, and the cell extracts were subjected to SDS-PAGE followed by immunoblotting with antibodies to phosphoserine 473 PKB from myotubes (*A*, *B*, *D*, *E*) or myoblasts (*C*), phosphoserine 21 (GSK3α), and phosphoserine 9 (GSK3β) (*D*), or phospho-ERK1/2 (*E*). Aliquots were also subjected to blotting with antibodies to PKB, GSK3 or ERK1/2. *B*, *left*, serine phosphorylation of PKB in myotubes pretreated or not with 0.1 mg/ml HGA was analyzed by densitometry. Results are expressed as % of the non-stimulated condition ± S.D. *B*, *right*, PKB activity was measured using Crosstide as substrate according to "Experimental Procedures." Results are expressed as arbitrary units. The experiments shown are representative of five (*A–D*) and six (*E*) independent experiments. *B*, *, $p < 0.05$; **, $p < 0.01$ *versus* control insulin-stimulated group.

exposed L6 myotubes, while it marginally decreased basal and insulin-stimulated glucose uptake (Fig. 9*A*). Similarly, with BDM treatment insulin-stimulated glycogen synthase activity was not affected by exposure to HGA for 24 h (Fig. 9*B*). Therefore, it would appear that PKC mediates the alterations in insulin signaling and action that result from exposure to HGA. Previous studies have implicated conventional and novel PKC isoforms in the development of insulin resistance. These negative effects are thought to be due to serine phosphorylation of the insulin receptor (51–53) or of IRS-1/2 (44–47). To investigate the mechanisms responsible for HGA-induced impairment of glucose metabolism in skeletal muscle cells, we determined

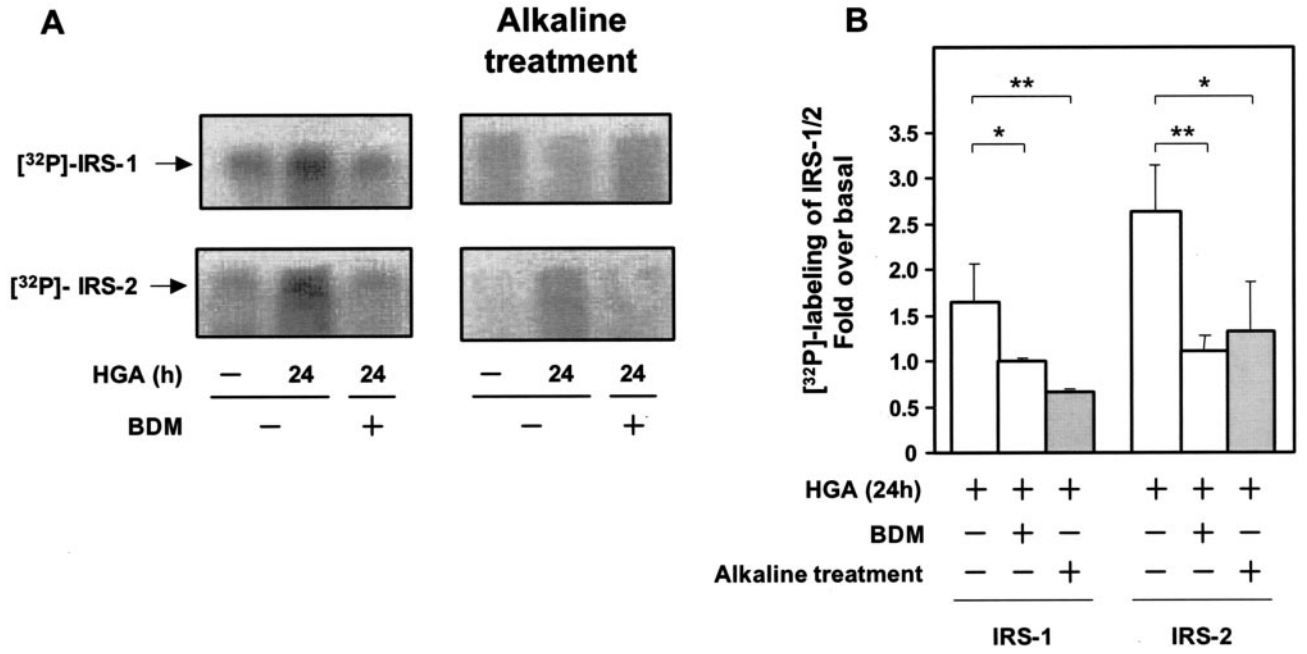

FIG. 6. **Effect of HGA on IRS-1 and IRS-2 Ser/Thr phosphorylation in L6 myotubes.** L6 myotubes, incubated with 0.1 mg/ml HGA for 24 h in the absence or the presence of 100 nM BDM as indicated, were labeled with [^{32}P]orthophosphate for the last 12 h. The cell lysates were immunoprecipitated with antibodies to IRS-1 or to IRS-2. Immunoprecipitated proteins were separated by SDS-PAGE and ^{32}P-proteins revealed by autoradiography. Potassium hydroxide treatment of the gels was performed as described under "Experimental Procedures." In *A*, the autoradiographs shown are representative of four independent experiments. In *B*, the means ± S.D. of the radioactivity of IRS-1/2 before and after alkaline treatment are shown. *, $p < 0.05$; **, $p < 0.01$ *versus* control HGA-pretreated group.

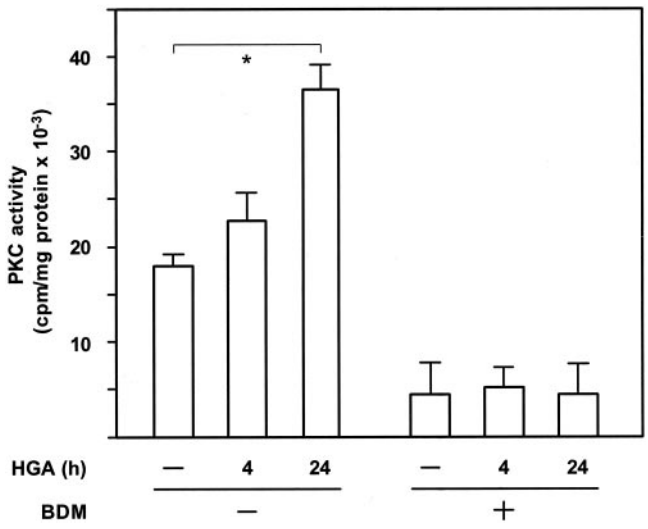

FIG. 7. **Effect of HGA on PKC activity in L6 myotubes.** L6 myotubes were incubated with 0.1 mg/ml HGA for the indicated times in the absence or presence of 100 nM BDM, as described under "Experimental Procedures." The cells were then solubilized and PKC activity measured in the cell lysates as described under "Experimental Procedures." *Bars* represent the mean ± S.D. of duplicate determinations of four independent experiments. *, $p < 0.01$ *versus* control non HGA- and non BDM-pretreated group.

the activities of PKC isoforms associated with diabetic complications (PKCβII) (54) and with inhibition of insulin receptor signaling (PKCα) (55), or which contribute to the stimulation of GLUT4 translocation (PKCζ) (5). As shown in Fig. 10*A*, following 24 h of stimulation with 0.1 mg/ml HGA, no modifications in enzyme activities of the βII and ζ isoforms of PKC were observed. However, an elevated activity and increased serine phosphorylation of the PKCα isoform was found after 12 h of incubation (Fig. 10*B*), corresponding with the time of maximum HGA action on IRS-1 and IRS-2 phosphorylation. We conclude that the negative effects of HGA on insulin metabolism signaling are specifically mediated by PKCα.

The stimulation of cells with AGEs has been shown to produce reactive oxygen species (56). Moreover, at high concentrations (1 mg/ml) HGA could induce NF-κB activation (18). To elucidate the roles of reactive oxygen species production in PKCα activation by HGA, we investigated whether the antioxidant could modulate activation of PKCα. L6 cells were treated with HGA (0.1 mg/ml) in the absence and presence of antioxidant PDTC (200 μM), which is described as inhibiting inducible nitric oxide synthase expression in RAW 264.7 macrophages treated with AGEs (57). As shown in Fig. 11*A*, PKCα activation was not influenced by PDTC addition. As expected, addition of PDTC inhibits ERK1/2 activation, previously demonstrated as activated by AGEs via a ROS-dependent mechanism (58). This result was confirmed by the inhibition of ROS production, after stimulation of cells with H_20_2 in the presence of PDTC (Fig. 11*B*). Moreover, similar inhibition of ROS production was observed in C_2C_{12} skeletal muscle cells treated by etomoxir in the presence of this inhibitor (59).

DISCUSSION

The present study shows, for the first time to the best of our knowledge, that HGA impairs insulin activation of glucose transport and of glycogen synthase in cultured L6 skeletal muscle myotubes. HGA action requires 4–12 h of exposure to HGA concentrations of at least 0.1 mg/ml and persists at 24 h. The concentration of HGA used throughout our study has been used previously by several investigators to study other HGA-mediated responses such as the regulation of vascular endothelial growth factor expression (20, 60), the redox-responsive transcription factors NF-κB and AP-1 (18) and the inducible nitric-oxide synthase (iNOS) expression in macrophages (57). The specificity of HGA action on the insulin-stimulated glucose metabolism was demonstrated by the lack of effect observed when the muscle cells were pretreated by nonglycated human albumin rather than HGA. Taken together, our findings suggest that HGA may cause, or at least participate in, skeletal

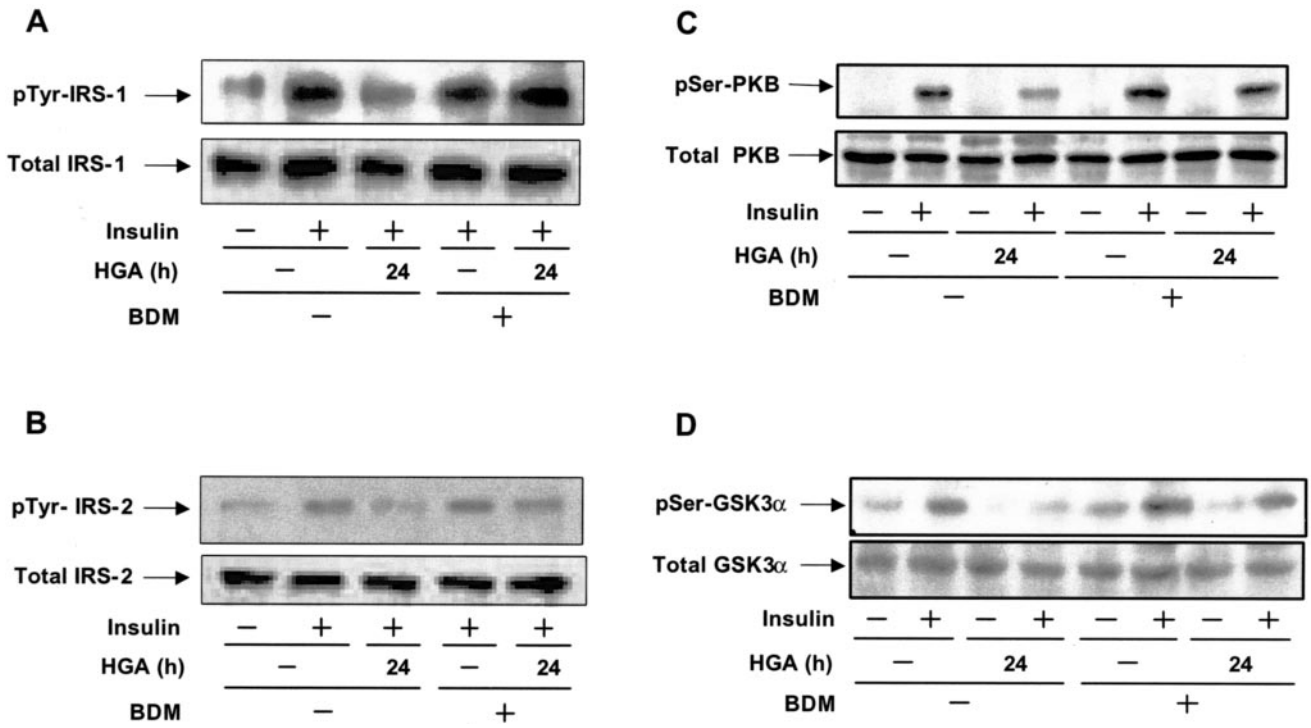

FIG. 8. **Effect of HGA on IRS-1/2, PKB, and GSK-3α phosphorylations in L6 myotubes upon blockade of PKC.** L6 myotubes were incubated with 0.1 mg/ml HGA for 24 h in the absence or the presence of 100 nM BDM, as described under "Experimental Procedures," and then stimulated with 100 nM insulin for 10 min. The cells were then solubilized and phosphorylation of IRS-1 (*A*), IRS-2 (*B*), PKB (*C*), and GSK3α (*D*) were analyzed. The immunoblots shown are representative of four (*A* and *B*) and three (*C* and *D*) independent experiments.

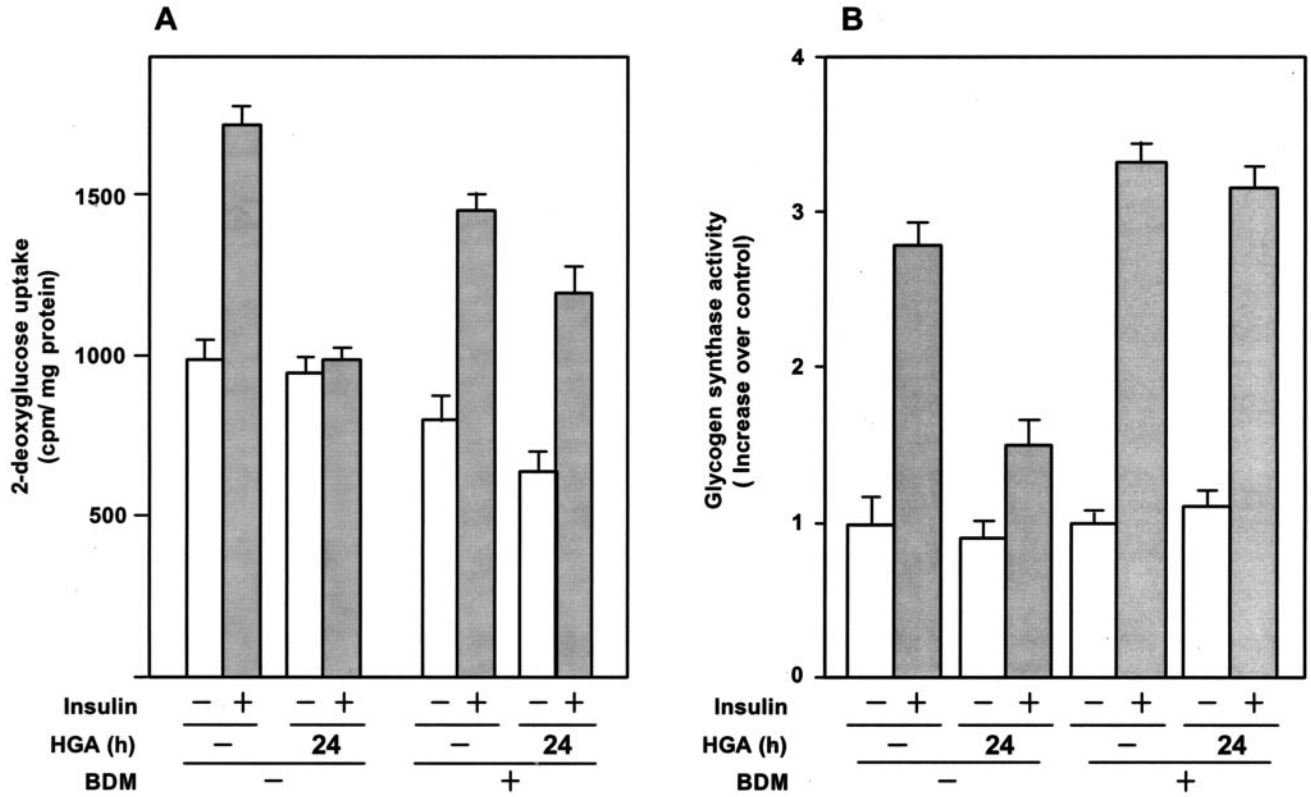

FIG. 9. **Effect of HGA on 2-DG uptake and glycogen synthase activity in L6 myotubes upon blockade of PKC.** L6 myotubes were incubated with 0.1 mg/ml HGA for 24 h in the presence or absence of 100 nM BDM, and then stimulated with 100 nM insulin for a further 10 min. The cells were then assayed for 2-DG uptake (*A*) or glycogen synthase activity (*B*) as described under "Experimental Procedures." *Bars* represent the mean ± S.D. of duplicate determinations in three (*A*) and four (*B*) independent experiments.

muscle insulin resistance at the level of glucose metabolism *in vivo*.

Exposure of L6 myotubes to HGA did not affect either the insulin receptor protein level or insulin-induced receptor kinase activation. Consistent with this, hormone-induced phosphorylation of Shc occurred at comparable levels in both the

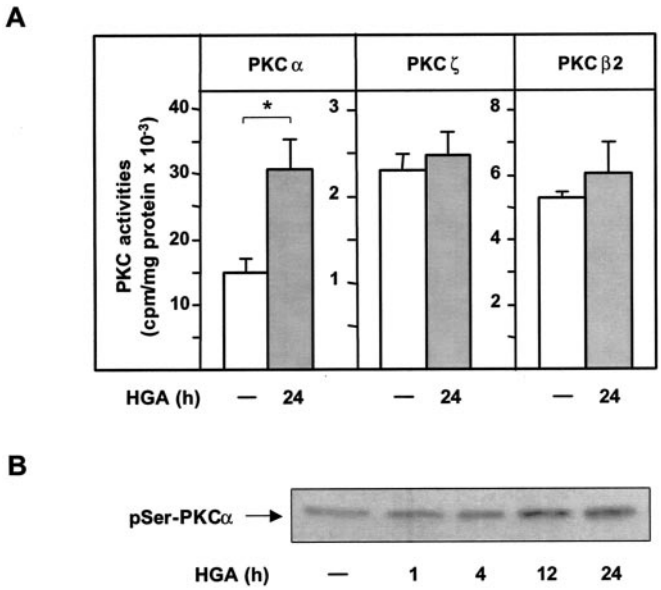

FIG. 10. **Effect of HGA on the activity of PKC isoforms in L6 myotubes.** L6 myotubes were incubated in the absence or presence of 0.1 mg/ml HGA for the indicated times. *A*, cells were solubilized and immunoprecipitated with isoform-specific PKC antibodies. The activity of PKC isoforms present in the immunoprecipitates were assayed as described under "Experimental Procedures." *B*, cell lysates were subjected to SDS-PAGE followed by immunoblotting with antibodies specific to the phosphorylated form of PKCα isoform. *Bars* represent the mean ± S.D. of duplicate determinations of four independent experiments. *A*, *, $p < 0.01$ *versus* control non-HGA-pretreated group.

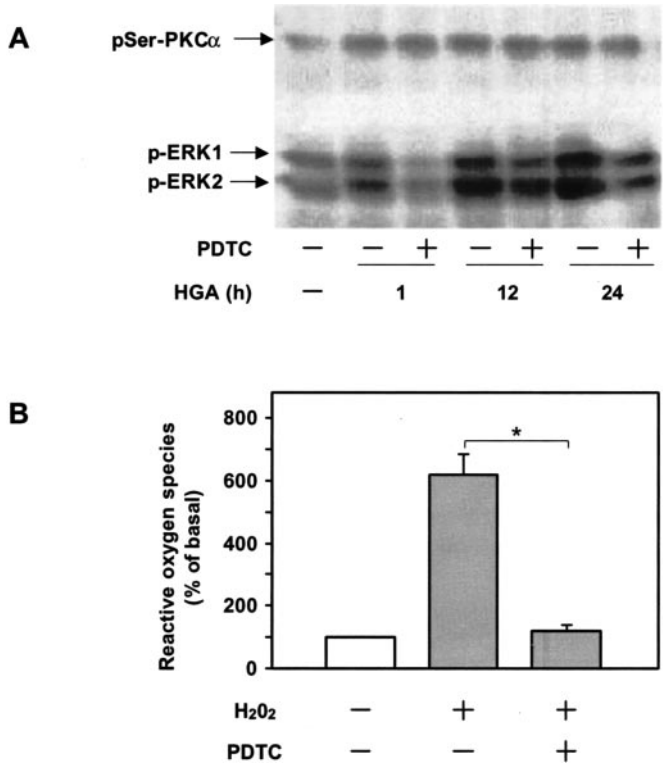

FIG. 11. **Effect of HGA on PKCα and ERK1/2 activation in the presence of PDTC in L6 myotubes.** *A*, L6 myotubes were preincubated with 200 μM PDTC for 30 min and then incubated with 0.1 mg/ml HGA for 1, 4, and 24 h. The cells were solubilized, and the cell extracts were subjected to SDS-PAGE followed by immunoblotting with antibodies either specific to the phosphorylated form of PKCα, to determine PKCα activation (*upper blot*) or to phospho-ERK1/2 (*lower blot*). Results shown are representative of three independent experiments. *B*, the cells were preincubated or not with 200 μM PDTC for 30 min and then incubated for 15 min in presence of H_2O_2 (500 μM). ROS were determined with the fluorescent probe CM-DCF. Results shown are representative of five independent experiments performed in triplicate. *, $p < 0.001$ *versus* non-PDTC-pretreated group.

presence and absence of HGA. However, the insulin-induced tyrosine phosphorylation of IRS-1 was reduced, and that of IRS-2 was almost completely abolished upon incubation with HGA for 12 h. These decreases in tyrosine-phosphorylated IRS proteins were not caused by impaired IRS synthesis or degradation since IRS-1 and IRS-2 protein expression levels were unchanged after HGA treatment of cells. Tyrosine dephosphorylation was also unlikely to cause the reduction in IRS phosphorylation. Indeed, no difference in phosphotyrosine phosphatase activity was detected either in total cell extracts or in immunoprecipitated IRS proteins from HGA-treated *versus* untreated cells (data not shown). Thus, in L6 myotubes, which are chronically exposed to HGA, IRS-1, and IRS-2 appear to have a reduced ability to be tyrosine-phosphorylated by the activated insulin receptor kinase.

In HGA-treated cells, decreased IRS-1/2 tyrosine phosphorylation was associated with increased phosphorylation of IRS-1/2 on serine/threonine residues and with increased activation of cellular PKC function. Importantly, inhibition of PKC activity appears to prevent this HGA-induced increase in serine/threonine phosphorylation and the decrease in tyrosine phosphorylation of IRS-1/2. This indicates that (i) HGA-induced serine/threonine phosphorylation of IRS-1/2 by PKC inhibits tyrosine phosphorylation of the proteins by the insulin receptor kinase and (ii) HGA impairs insulin-dependent IRS tyrosine phosphorylation by activating PKC signaling.

Previous studies using specific inhibitors of PKC isoforms have demonstrated that the effects of HGA on the development of diabetic nephropathy result from the glycated albumin-induced activation of mesengial PKCβII isoform which leads to the stimulation of collagen IV production (61, 62). The same PKC isoform has been implicated in vascular dysfunction (63) and development of retinopathy (64). Other studies have shown that PKC activation desensitizes insulin signaling by increasing serine/threonine phosphorylation of IRS-1 and IRS-2 (46–49). Our present study demonstrates that increased serine/threonine IRS phosphorylation by PKC can be triggered by chronic exposure to HGA and is accompanied by decreased insulin signaling. While there is also evidence that PKCs phosphorylate the insulin receptor leading to inhibition of insulin signaling (51–53), our findings in L6 myotubes demonstrate that HGA does not alter insulin receptor autophosphorylation or kinase activity. The decrease in insulin-induced tyrosine phosphorylation of IRS-1 and IRS-2 caused by HGA was accompanied by a decrease in insulin-stimulated PI3K activity, PKB activity, serine phosphorylation of both GSK3 isoforms and by a reduced stimulation of glucose uptake and glycogen synthase activity. As is the case for insulin-induced tyrosine phosphorylation of IRS-1/2, the effect of HGA on all of these downstream events was prevented by inhibition of PKC activity. Interestingly, IRS-Grb2 association was unchanged in cells exposed to HGA, indicating that HGA differentially affects phosphorylation of the tyrosine binding sites on the insulin receptor substrate p85 and Grb2. Consistent with this finding and with the unchanged phosphorylation of Shc in HGA-exposed *versus* unexposed cells, insulin activation of ERK1/2 was also unaffected by HGA. Similarly, the effect of insulin on thymidine incorporation was identical in cells exposed or not to HGA. Thus, HGA induces resistance to insulin action specifically in metabolic responses but not in proliferative ones, and these effects are independent of the differentiation status of the muscle cells. Interestingly, our observations are supported by a recent study showing that restricted intake of dietary glycoxi-

dation products improves insulin sensitivity of metabolic responses in db/db mice (65).

HGA could specifically stimulate certain PKC isoforms, resulting in the selectively increased serine/threonine phosphorylation of the IRS proteins as well as of the insulin receptor. However, as previously shown in PKC-transfected cells, an increase in PKC activity to levels similar to those occurring in HGA-exposed cells may not be sufficient to inhibit the insulin receptor kinase, while being fully capable of blocking IRS function (29, 53). Several studies have shown that the PKC family of serine/threonine kinases may be implicated in development of insulin resistance. The PKC family is composed of at least 11 isoforms, which are categorized into three groups according to their structure and mechanisms of activation. The atypical isoforms (λ and ζ) appear to play a stimulatory role in the regulation of glucose transport. In contrast, isoforms of conventional and novel groups appear to be implicated in generation of reduced insulin sensitivity. Indeed, PKCα, β, and θ have been shown to inhibit insulin receptor tyrosine phosphorylation (66). Our findings demonstrate that HGA pretreatment induces a selective activation of PKCα. The intracellular signaling mechanisms by which HGA induces PKCα activation remain to be determined, however, our results obtained after stimulation of PKCα in the presence of PDTC, a potent antioxidant and inhibitor of NF-κB, show that this pathway is independent of ROS production. This observation, in contrast to the induction of iNOS expression in macrophages after AGEs treatment (57), could be explained by the "early glycation products" nature of human glycated albumin and by the low concentration used in this study. PKCα appears to phosphorylate IRS-1 and IRS-2 on serine/threonine residues. These serine/threonine phosphorylations would then lead to a reduced interaction between the IRS proteins and the regulatory p85 subunit of PI3K without affecting the interaction between IRS and Grb2. To our knowledge, the present study provides the first indication that IRS serine/threonine phosphorylation by PKCα specifically induces an inhibition of insulin-stimulated glucose metabolism that is triggered by chronic exposure to HGA. This specific inhibition of glucose metabolism occurs without changes in growth-related pathways regulated by the hormone.

Hyperglycemia is thought to activate cellular PKCs through direct and indirect mechanisms (46–48). Chronic hyperglycemia also leads to impaired insulin responsiveness in the hormone target tissues (46, 48, 53), and is associated with most diabetic complications (53, 67). While the molecular basis of long-term diabetic complications is complex, glucose-induced insulin resistance has been linked to an increased flux of glucose through the hexosamine biosynthetic pathway (68–72). In addition, in the present report, we show that HGA induces resistance to insulin at the IRS level by activating the PKCα isoform. Therefore, HGA and hence AGE products, which result from chronic hyperglycemia, may participate in the development of insulin resistance.

Further studies are needed to determine the mechanisms of PKCα activation and the identification of IRS-1 and IRS-2 serine/threonine residues phosphorylated by PKCα implicated in the impairment in insulin signaling after HGA treatment.

Acknowledgments—We thank Drs. Anne Johnston and Sarah Longnus for discussion and critical reading of the article and Dr. Kientsch-Engels (Roche AG) for the determination of the CML concentrations in our human glycated and nonglycated albumin preparations.

REFERENCES

1. Pessin, J. E., and Saltiel, A. R. (2000) *J. Clin. Investig.* **106,** 165–169
2. Van Obberghen, E., Baron, V., Delahaye, L., Emanuelli, B., Filippa, N., Giorgetti-Peraldi, S., Lebrun, P., Mothe-Satney, I., Peraldi, P., Rocchi, S., Sawka-Verhelle, D., Tartare-Deckert, S., and Giudicelli, J. (2001) *Eur J. Clin. Investig.* **31,** 966–977
3. White, M. F. (1998) *Mol. Cell Biochem.* **182,** 3–11
4. Alessi, D. R., and Cohen, P. (1998) *Curr. Opin. Genet. Dev.* **8,** 55–62
5. Standaert, M. L., Galloway, L., Karnam, P., Bandyopadhyay, G., Moscat, J., and Farese, R. V. (1997) *J. Biol. Chem.* **272,** 30075–30082
6. Farese, R. V. (2002) *Am. J. Physiol. Endocrinol. Metab.* **283,** E1–E11
7. Kahn, B. B., and Flier, J. S. (2000) *J. Clin. Investig.* **106,** 473–481
8. Hager, S. R., Jochen, A. L., and Kalkhoff, R. K. (1991) *Am. J. Physiol. Endocrinol. Metab.* **260,** E353–E362
9. Davidson, M. B., Bouch, C., Venkatesan, N., and Karjala, R. G. (1994) *Am. J. Physiol. Endocrinol. Metab.* **267,** E808–E813
10. Idris, I., Gray, S., and Donnelly, R. (2002) *Ann. N. Y. Acad. Sci.* **967,** 176–182
11. Yerneni, K. K., Bai, W., Khan, B. V., Medford, R. M., and Natarajan, R. (1999) *Diabetes* **48,** 855–864
12. Nishikawa, T., Edelstein, D., Du, X. L., Yamagishi, S., Matsumura, T., Kaneda, Y., Yorek, M. A., Beebe, D., Oates, P. J., Hammes, H. P., Giardino, I., and Brownlee, M. (2000) *Nature* **404,** 787–790
13. Degenhardt, T. P., Thorpe, S. R., and Baynes, J. W. (1998) *Cell Mol. Biol.* **44,** 1139–1145
14. Brownlee, M. (2000) *Metabolism* **49,** 9–13
15. Wells-Knecht, K. J., Zyzak, D. V., Litchfield, J. E., Thorpe, S. R., and Baynes, J. W. (1995) *Biochemistry* **34,** 3702–3709
16. Thornalley, P. J. (1990) *Biochem. J.* **269,** 1–11
17. Brownlee, M. (2001) *Nature* **414,** 813–820
18. Hattori, Y., Suzuki, M., Hattori, S., and Kasai, K. (2002) *Hypertension* **39,** 22–28
19. Ruggiero-Lopez, D., Rellier, N., Lecomte, M., Lagarde, M., and Wiernsperger, N. (1997) *Diabetes Res. Clin. Pract.* **34,** 135–142
20. Treins, C., Giorgetti-Peraldi, S., Murdaca, J., and Van Obberghen, E. (2001) *J. Biol. Chem.* **276,** 43836–43841
21. Yamagishi, S.-i., Inagaki, Y., Okamoto, T., Amano, S., Koga, K., Takeuchi, M., and Makita, Z. (2002) *J. Biol. Chem.* **277,** 20309–20315
22. Hoffmann, S., Friedrichs, U., Eichler, W., Rosenthal, A., and Wiedemann, P. (2002) *Graefes Arch Clin Exp Ophthalmol* **240,** 996–1002
23. Makita, Z., Yanagisawa, K., Kuwajima, S., Yoshioka, N., Atsumi, T., Hasunuma, Y., and Koike, T. (1995) *J. Diabetes Complic.* **9,** 265–268
24. Flyvbjerg, A. (2000) *Diabetologia* **43,** 1205–1223
25. Schmidt, A. M., Yan, S. D., Yan, S. F., and Stern, D. M. (2000) *Biochim. Biophy. Acta, Mol. Cell Res.* **1498,** 99–111
26. Wu, V.-Y., Shearman, C. W., and Cohen, M. P. (2001) *Biochem. Biophys. Res. Commun.* **284,** 602–606
27. Naitoh, T., Kitahara, M., and Tsuruzoe, N. (2001) *Cell. Signal.* **13,** 331–334
28. Peraldi, P., and Spiegelman, B. (1998) *Mol. Cell Biochem.* **182,** 169–175
29. Formisano, P., Oriente, F., Fiory, F., Caruso, M., Miele, C., Maitan, M. A., Andreozzi, F., Vigliotta, G., Condorelli, G., and Beguinot, F. (2000) *Mol. Cell Biol.* **20,** 6323–6333
30. Pongor, S., Ulrich, P. C., Bencsath, F. A., and Cerami, A. (1984) *Proc. Natl. Acad. Sci. U. S. A.* **81,** 2684–2688
31. Morcos, M., Sayed, A. A. R., Bierhaus, A., Yard, B., Waldherr, R., Merz, W., Kloeting, I., Schleicher, E., Mentz, S., Abd el Baki, R. F., Tritschler, H., Kasper, M., Schwenger, V., Hamann, A., Dugi, K. A., Schmidt, A.-M., Stern, D., Ziegler, R., Haering, H. U., Andrassy, M., van der Woude, F., and Nawroth, P. P. (2002) *Diabetes* **51,** 3532–3544
32. Fields, R. (1971) *Biochem. J.* **124,** 581–590
33. Smith, R., and MacQuarrie, R. (1978) *Anal. Biochem.* **90,** 246–255
34. Smith, P. R., and Thornalley, P. J. (1992) *Eur. J. Biochem.* **210,** 729–739
35. Scimeca, J. C., Ballotti, R., Nguyen, T. T., Filloux, C., and Van Obberghen, E. (1991) *Biochemistry* **30,** 9313–9319
36. Hajduch, E., Alessi, D. R., Hemmings, B. A., and Hundal, H. S. (1998) *Diabetes* **47,** 1006–1013
37. Formisano, P., Sohn, K., Miele, C., Di Finizio, B., Petruzziello, A., Riccardi, G., Beguinot, L., and Beguinot, F. (1993) *J. Biol. Chem.* **268,** 5241–5248
38. Filippa, N., Sable, C. L., Filloux, C., Hemmings, B., and Van Obberghen, E. (1999) *Mol. Cell Biol.* **19,** 4989–5000
39. Maziere, C., Floret, S., Santus, R., Morliere, P., Marcheux, V., and Maziere, J.-C. (2003) *Free Radic. Biol. Med.* **34,** 629–636
40. Xu, B., Chibber, R., Ruggerio, D., Kohner, E., Ritter, J., and Ferro, A. (2003) *FASEB J.* **17,** 1289–1291
41. Nagai, R., Matsumoto, K., Ling, X., Suzuki, H., Araki, T., and Horiuchi, S. (2000) *Diabetes* **49,** 1714–1723
42. Kurowski, T., Lin, Y., Luo, Z., Tsichlis, P., Buse, M., Heydrick, S., and Ruderman, N. (1999) *Diabetes* **48,** 658–663
43. Kim, Y., Peroni, O., Franke, T., and Kahn, B. (2000) *Diabetes* **49,** 847–856
44. De Fea, K., and Roth, R. A. (1997) *Biochemistry* **36,** 12939–12947
45. Jiang, Z., Zhou, Q., Chatterjee, A., Feener, E., Myers, M., White, M., and King, G. (1999) *Diabetes* **48,** 1120–1130
46. Nakajima, K., Yamauchi, K., Shigematsu, S., Ikeo, S., Komatsu, M., Aizawa, T., and Hashizume, K. (2000) *J. Biol. Chem.* **275,** 20880–20886
47. Valverde, A. M., Lorenzo, M., Navarro, P., Mur, C., and Benito, M. (2000) *FEBS Lett.* **472,** 153–158
48. Motley, E. D., Kabir, S. M., Eguchi, K., Hicks, A. L., Gardner, C. D., Reynolds, C. M., Frank, G. D., and Eguchi, S. (2001) *Cell. Mol. Biol. (Noisy-le-grand)* **47,** 1059–1062
49. Ravichandran, L. V., Esposito, D. L., Chen, J., and Quon, M. J. (2001) *J. Biol. Chem.* **276,** 3543–3549
50. Griffin, M., Marcucci, M., Cline, G., Bell, K., Barucci, N., Lee, D., Goodyear, L., Kraegen, E., White, M., and Shulman, G. (1999) *Diabetes* **48,** 1270–1274
51. Bossenmaier, B., Mosthaf, L., Mischak, H., Ullrich, A., and Haring, H. U. (1997) *Diabetologia* **40,** 863–866
52. Strack, V., Hennige, A. M., Krutzfeldt, J., Bossenmaier, B., Klein, H. H., Kellerer, M., Lammers, R., and Haring, H. U. (2000) *Diabetologia* **43,** 443–449
53. Tomas, E., Lin, Y. S., Dagher, Z., Saha, A., Luo, Z., Ido, Y., and Ruderman, N. B. (2002) *Ann. N. Y. Acad. Sci.* **967,** 43–51
54. Koya, D., and King, G. L. (1998) *Diabetes* **47,** 859–866

55. Chin, J. E., Liu, F., and Roth, R. A. (1994) *Mol Endocrinol* **8,** 51–58
56. Yan, S., Schmidt, A., Anderson, G., Zhang, J., Brett, J., Zou, Y., Pinsky, D., and Stern, D. (1994) *J. Biol. Chem.* **269,** 9889–9897
57. Wu, C.-H., Huang, C.-M., Lin, C.-H., Ho, Y.-S., Chen, C.-M., and Lee, H.-M. (2002) *Mol. Cell. Endocrinol.* **194,** 9–17
58. Yeh, C.-H., Sturgis, L., Haidacher, J., Zhang, X.-N., Sherwood, S. J., Bjercke, R. J., Juhasz, O., Crow, M. T., Tilton, R. G., and Denner, L. (2001) *Diabetes* **50,** 1495–1504
59. Cabrero, A., Alegret, M., Sanchez, R. M., Adzet, T., Laguna, J. C., and Carrera, M. V. (2002) *J. Biol. Chem.* **277,** 10100–10107
60. Mamputu, J.-C., and Renier, G. (2002) *J. Diabetes Compl.* **16,** 284–293
61. Cohen, M. P., Ziyadeh, F. N., Lautenslager, G. T., Cohen, J. A., and Shearman, C. W. (1999) *Am. J. Physiol. Renal Physiol.* **276,** F684–690
62. Scivittaro, V., Ganz, M. B., and Weiss, M. F. (2000) *Am. J. Physiol. Renal Physiol.* **278,** F676–683
63. Ido, Y., Chang, K. C., Lejeune, W. S., Bjercke, R. J., Reiser, K. M., Williamson, J. R., and Tilton, R. G. (2001) *Microcirculation* **8,** 251–263
64. Aiello, L. P. (2002) *Survey Ophthalmol.* **47,** S263-S269
65. Hofmann, S. M., Dong, H.-J., Li, Z., Cai, W., Altomonte, J., Thung, S. N., Zeng, F., Fisher, E. A., and Vlassara, H. (2002) *Diabetes* **51,** 2082–2089
66. Kellerer, M., Mushack, J., Seffer, E., Mischak, H., Ullrich, A., and Haring, H. U. (1998) *Diabetologia* **41,** 833–838
67. Rossetti, L., Giaccari, A., and DeFronzo, R. A. (1990) *Diabetes Care* **13,** 610–630
68. Hebert, L. F., Jr., Daniels, M. C., Zhou, J., Crook, E. D., Turner, R. L., Simmons, S. T., Neidigh, J. L., Zhu, J. S., Baron, A. D., and McClain, D. A. (1996) *J. Clin. Investig.* **98,** 930–936
69. Hawkins, M., Barzilai, N., Liu, R., Hu, M., Chen, W., and Rossetti, L. (1997) *J. Clin. Investig.* **99,** 2173–2182
70. Matsuoka, T., Kajimoto, Y., Watada, H., Kaneto, H., Kishimoto, M., Umayahara, Y., Fujitani, Y., Kamada, T., Kawamori, R., and Yamasaki, Y. (1997) *J. Clin. Investig.* **99,** 144–150
71. Rossetti, L. (2000) *Endocrinology* **141,** 1922–1925
72. McClain, D. A., Lubas, W. A., Cooksey, R. C., Hazel, M., Parker, G. J., Love, D. C., and Hanover, J. A. (2002) *Proc. Natl. Acad. Sci. U. S. A.* **99,** 10695–10699

Human Glycated Albumin Affects Glucose Metabolism in L6 Skeletal Muscle Cells by Impairing Insulin-induced Insulin Receptor Substrate (IRS) Signaling through a Protein Kinase Cα-mediated Mechanism

Claudia Miele, Audrey Riboulet, Maria Alessandra Maitan, Francesco Oriente, Chiara Romano, Pietro Formisano, Jean Giudicelli, Francesco Beguinot and Emmanuel Van Obberghen

J. Biol. Chem. 2003, 278:47376-47387.
doi: 10.1074/jbc.M301088200 originally published online September 11, 2003
