Original Article

Insulin Signaling in Human Visceral and Subcutaneous Adipose Tissue In Vivo

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In this study, we evaluated the activation of various insulin signaling molecules in human fat in vivo and compared signaling reactions in visceral and subcutaneous fat depots. Paired abdominal omental and subcutaneous fat biopsies were obtained from nonobese subjects with normal insulin sensitivity under basal conditions and 6 and 30 min following administration of intravenous insulin. Insulin receptor phosphorylation was more intense and rapid and insulin receptor protein content was greater in omental than in subcutaneous adipose tissue ($P < 0.05$). Insulin-induced phosphorylation of Akt also occurred to a greater extent and earlier in omental than in subcutaneous fat ($P < 0.05$) in the absence of significant changes in Akt protein content. Accordingly, phosphorylation of the Akt substrate glycogen synthase kinase-3 was more responsive to insulin stimulation in omental fat. Protein content of extracellular signal–regulated kinase (ERK)-1/2 was threefold higher in omental than in subcutaneous fat ($P < 0.05$), and ERK phosphorylation showed an early 6-min peak in omental fat, in contrast with a more gradual increase observed in subcutaneous fat. In conclusion, the adipocyte insulin signaling system of omental fat shows greater and earlier responses to insulin than that of subcutaneous fat. These findings may contribute to explain the biological diversity of the two fat depots. *Diabetes* 55:952–961, 2006

Insulin is central to the regulation of anabolic responses in fat. Insulin stimulates glucose and free fatty acid uptake, inhibits lipolysis, and stimulates de novo fatty acid synthesis in adipocytes. In addition, insulin modulates adipose tissue growth and differentiation. For example, both the expression levels and activities of two master adipose tissue transcriptional regulators, such as sterol regulatory element–binding protein-1 and peroxisome proliferator–activated receptor (PPAR)-γ, are under the control of insulin in adipocytes (1–4).

The biological actions of insulin in the adipose tissue are mediated by high-affinity cell-surface receptors with intrinsic tyrosine kinase activity (5). Insulin binding induces autophosphorylation and activation of the receptor tyrosine kinase, leading to the recruitment of substrate proteins, including the insulin receptor substrate (IRS) proteins, which signal both metabolic and mitogenic processes, and the Src-homology collagen (Shc) proteins, which are primarily coupled to mitogenic effects (6). Following these initial tyrosine phosphorylation reactions, insulin signals are conveyed to multiple intracellular lipid and serine/threonine kinases and thus coupled to specific biological responses. Activation of phosphatidylinositol (PI) 3-kinase and Akt mediates insulin stimulation of glucose uptake and GLUT4 vesicle translocation to the plasma membrane (7,8) and is involved in various other insulin effects, including inhibition of lipolysis and activation of fatty acid, glycogen, protein, and DNA synthesis (9). On the other hand, activation of the extracellular signal–regulated kinase (ERK) pathway is implicated in mitogenic responses of preadipocytes to growth factors (10), serine phosphorylation of sterol regulatory element–binding protein (11), and both expression and activation of PPAR-γ (3,4).

An excessive amount of adipose tissue is causally linked to the development of type 2 diabetes, premature atherosclerosis, and cardiovascular disease (12). The complex relationship between excess adiposity and enhanced risk of developing these health complications has not been fully elucidated, but it is increasingly apparent that accumulation of adipose tissue in the abdominal region, rather than excess body fat per se, is the major player in the adverse metabolic consequences of obesity (13). Support for a specific detrimental role of abdominal and in particular visceral fat also comes from studies in insulin-resistant humans and rats, in which surgical removal of visceral fat was followed by marked metabolic improvement (14–16). Conversely, the amount of intra-abdominal fat is positively associated with greater risk of developing type 2 diabetes (17).

Visceral fat is known to exhibit morphological and functional differences in comparison with subcutaneous fat, including differences of insulin action on regulation of lipolysis (18). The observation that insulin exerts weaker anti-lipolytic effects in adipocytes isolated from visceral compared with subcutaneous fat depots (19) has sug-

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ERK, extracellular signal–regulated kinase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GSK, glycogen synthase kinase; IRS, insulin receptor substrate; ITT, insulin tolerance test; MAP, mitogen-activated protein; PAI-1, plasminogen activator inhibitor 1; PI, phosphatidylinositol; PPAR, peroxisome proliferator–activated receptor.

gested that visceral fat may possess a less-efficient intracellular insulin signaling system and thus behave as a relatively insulin-resistant tissue. However, most data in human adipose tissue derive from studies carried out in isolated adipocytes in vitro. Information on insulin signaling reactions in human adipose tissue depots in vivo is not available.

In this study, we used a novel experimental protocol involving injection of an intravenous insulin bolus in normal volunteers undergoing surgery and attainment of multiple fat biopsies at various times and from different fat depots. With this approach, we were able to detect the activation of the insulin signaling cascade in adipose tissue as it occurs in vivo following insulin stimulation and to compare signaling reactions in the visceral and subcutaneous fat depots from the same individual. We show that visceral fat is characterized by higher expression levels of specific insulin signaling proteins and more pronounced and earlier activation of the insulin receptor, Akt, glycogen synthase kinase (GSK)-3, and ERK-1/2 in response to insulin.

RESEARCH DESIGN AND METHODS

Adipose tissue biopsies. Paired abdominal subcutaneous and omental fat biopsies were obtained from seven nonobese subjects with normal glucose tolerance who underwent elective open abdominal surgery for nonmalignant diseases (i.e., colecystectomy, inguinal hernia, or hysterectomy). The subjects were all healthy and took no regular medications. All subjects gave their informed consent before the surgical procedure. The study protocol was approved by the Independent Ethical Committee of the Azienda Ospedaliera Policlinico Consorziale, University of Bari School of Medicine, Bari, Italy. Two days before the surgical procedure, all subjects underwent anthropometric and metabolic characterization, including height and weight measurements, fasting blood chemistry, body composition measurements by bioelectric impedance analysis, and assessment of insulin sensitivity. Insulin sensitivity was estimated by an insulin tolerance test (ITT) using 0.1 unit/kg regular insulin, as previously described (20).

On the day of surgery, after an overnight fast, general anesthesia was produced at 9:00 A.M. Adipose tissue specimens from the subcutaneous and omental adipose tissue regions were obtained under basal conditions and 6 and 30 min following administration of regular insulin in an antecubital vein (Humulin R 0.1 unit/kg i.v.; Eli Lilly, Indianapolis, IN). Plasma glucose levels were clamped at 5.5 mmol/l using an intravenous glucose infusion throughout the study. Approximately 1–3 g adipose tissue was obtained from each fat depot and at each time point. All biopsies were handled under sterile conditions and immediately frozen in liquid N_2 for subsequent procedures. DNA was extracted from 100–200 mg frozen adipose tissue with a commercially available DNA purification kit (Promega, Madison, WI) and quantified by spectrophotometry at 260 nm. For cell-size analysis, mature adipocytes were isolated from subcutaneous and visceral biopsies as described (21). Digital images of the fat cells, obtained under light microscopy, were analyzed on a personal computer using the public domain image analysis program Scion, available at www.scioncorp.com, according to published protocols (22).

Cell culture. 3T3-L1 fibroblasts (American *Type Culture* Collection, Rockville, MD) were grown and differentiated into adipocytes as described previously (21). Upon full differentiation, cells were stimulated with 100 nmol/l recombinant human insulin (Roche Diagnostics, Mannheim, Germany) for the indicated times or left untreated. For preparing total cell lysates, 3T3-L1 adipocytes were washed with Ca^{2+}/Mg^{2+}-free PBS and then mechanically detached in ice-cold lysis buffer containing 50 mmol/l HEPES (pH 7.5), 150 mmol/l NaCl, 1 mmol/l $MgCl_2$, 1 mmol/l $CaCl_2$, 10% glycerol, 10 mmol/l sodium pyrophosphate, 10 mmol/l sodium fluoride, 2 mmol/l EDTA, 2 mmol/l phenylmethylsulfonyl fluoride, 5 μg/ml leupeptin, 2 mmol/l sodium orthovanadate, and 1% Nonidet P-40. After incubation for 45 min at 4°C, the preparation was centrifuged at 12,000*g* for 10 min at 4°C. The resulting supernatant was assayed for determination of protein concentration using the Bradford method.

Analytical procedures. Plasma glucose was determined by the glucose-oxidase method (Sclavo, Siena, Italy). Serum insulin was measured using a human insulin radioimmunoassay (Linco, St. Charles, MO). White blood cell count was measured in the local laboratory by an automated cell counter. Body composition was estimated in the fasting state by bioelectrical impedance analysis using a tetrapolar device (BIA 101/S; RJL Systems, Detroit, MI; and Akern s.r.l.; Akern, Florence, Italy). The fat-free mass was calculated by the Heitmann's equation (23), and fat mass was calculated as the difference between body weight and fat-free mass.

Immunoprecipitation and immunoblotting. The frozen adipose tissue was powdered in a stainless-steel mortar and pestle with liquid N_2 and homogenized for 30 s with an Ultra-Turrax (Janke & Kunkel, IKA-Werk, Staufen, Germany) in ice-cold lysis buffer. The tissue homogenate was incubated for 45 min at 4°C with gentle stirring and then centrifuged at 100,000*g* for 60 min. The resulting supernatant was collected and assayed for protein concentration using the Bradford dye binding assay kit. Equal amounts of adipose tissue extracts (1 mg) were subjected to immunoprecipitation with anti–IRS-1, anti–IRS-2, or anti-Shc antibodies overnight at 4°C. The resulting immune complexes were adsorbed to protein A-Sepharose beads for 2 h at 4°C, washed three times with lysis buffer, and then eluted with laemmli buffer for 5 min at 100°C. For immunoblotting studies, equal amounts of solubilized adipose tissue or 3T3-L1 adipocyte proteins were resolved by electrophoresis on 7 or 10% SDS polyacrylamide gels, as appropriate. The resolved proteins were electrophoretically transferred to nitrocellulose or polyvinylidine fluoride membranes (Hybond-ECL; Amersham Life Science, Arlington Heights, IL) using a transfer buffer containing 192 mmol/l glycine, 20% (vol/vol) methanol, and 0.02% SDS, and incubated overnight at 4°C with the indicated antibodies, as previously described (21). The proteins were visualized by enhanced chemiluminescence using horseradish peroxidase–labeled anti-rabbit or anti-mouse IgG (Amersham Life Science) and quantified by densitometric analysis using Quantity One image analysis software (Bio-Rad, Hercules, CA).

Antibodies. Polyclonal anti–glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and anti–insulin receptor β-subunit and monoclonal anti-phosphotyrosine antibodies (PY99) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Polyclonal anti-Akt, anti–phospho-Akt (Thr-308), anti–phospho-Akt (Ser-473), anti-MEK1/2, anti–phospho-p42/44 mitogen-activated protein (MAP) kinase (ERK-1/2) (Thr-202/Tyr-204), anti–phospho–GSK-3α/β (Ser-9/Ser-21), and anti–Elk-1 were from Cell Signaling Technology (Beverly, MA). Polyclonal anti-Shc, anti–IRS-1, anti–IRS-2, anti–PI 3-kinase p85, and anti–GSK-3 antibodies were purchased from Upstate (Lake Placid, NY). Anti-MAP kinase (ERK-1/2) antibodies were from Zymed Laboratories (San Francisco, CA).

Statistical analyses. For the evaluation of changes in the phosphorylation levels of the proteins assessed, one-way ANOVA and Tukey's post hoc tests were used. Differences between the omental and subcutaneous sample groups were analyzed by the Student's *t* test for independent samples. All data are expressed as the means ± SE. *P* values <0.05 were considered to represent statistical significance.

RESULTS

Activation of insulin signaling in human adipose tissue in vivo. To evaluate the activation of the insulin signal transduction system in human adipose tissue in vivo, regular insulin was administered as an intravenous bolus injection to individuals undergoing elective surgery for nonmalignant diseases. This procedure was carried out under anesthesia and with glucose levels maintained constantly at 5.5 mmol/l by infusing a glucose solution. The insulin dose (0.1 unit/kg) utilized to investigate signaling reactions in fat was chosen to be equal to the dose used in the ITT to assess insulin sensitivity. Fat biopsies from abdominal subcutaneous and omental fat depots were obtained at multiple times before and after insulin injection and were processed to yield tissue protein extracts for immunoblotting analyses, as described in RESEARCH DESIGN AND METHODS. Figure 1*A* shows a phosphotyrosine immunoblot of subcutaneous and omental fat proteins at baseline and 6 and 30 min following intravenous insulin injection. Insulin administration resulted in enhanced tyrosine phosphorylation of molecular species of 95 kDa, corresponding to the insulin receptor β-subunit, and 175 kDa, corresponding to the IRS proteins. In the omental fat, peak phosphorylation of these proteins occurred at 6 min with much lower levels observed at 30 min after the injection, whereas in the subcutaneous fat the tyrosine phosphoproteins were stimulated only slightly at 6 min and further at

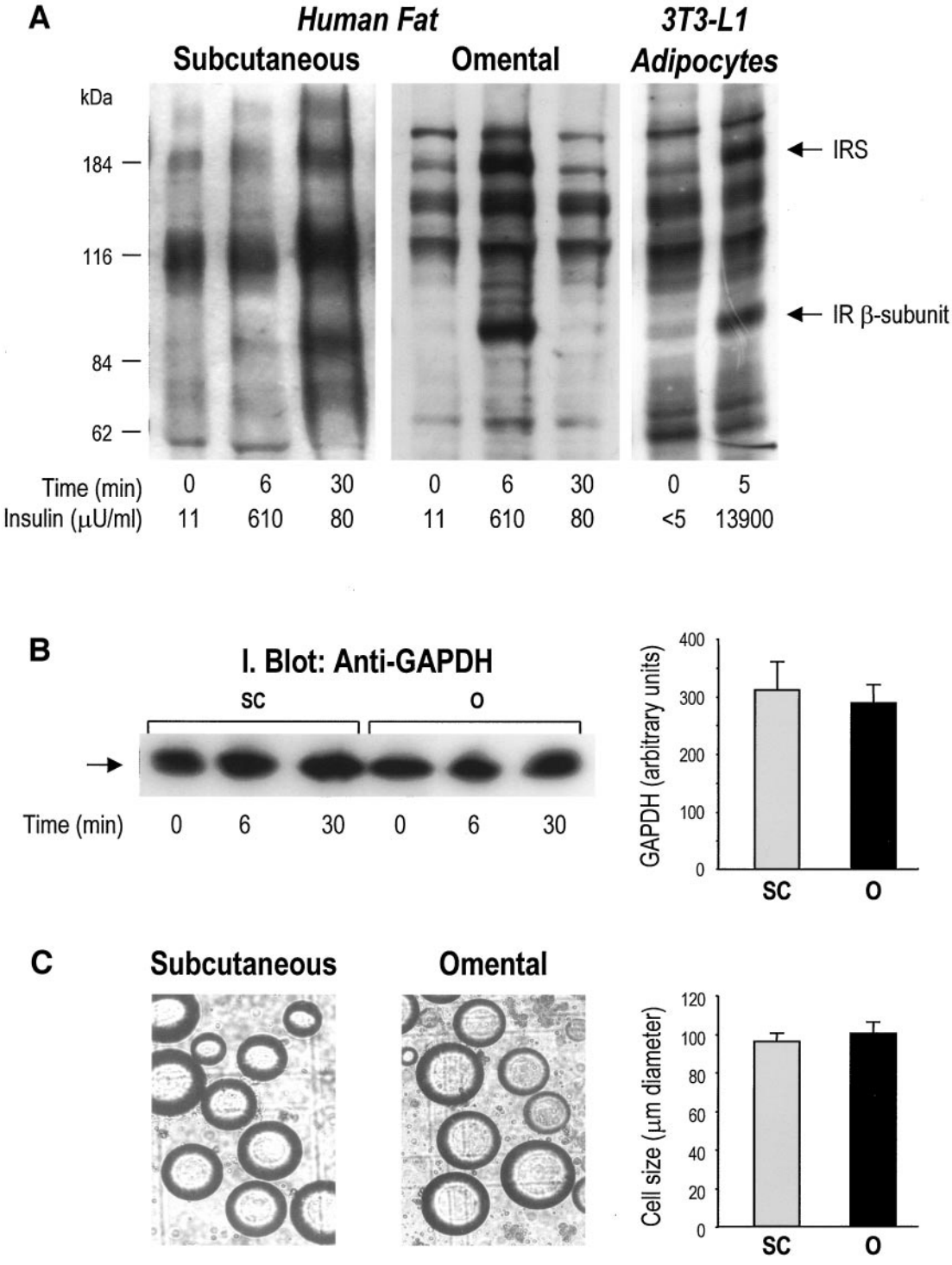

FIG. 1. ***A*: Activation of insulin signaling in human adipose tissue in vivo. One subject undergoing elective surgery was stimulated with 0.1 unit/kg insulin as an intravenous bolus injection. Subcutaneous and omental fat biopsies were obtained at baseline and 6 and 30 min following insulin injection. Fat protein extracts were analyzed by immunoblotting with anti-phosphotyrosine antibodies, as described in RESEARCH DESIGN AND METHODS. For comparison, a phosphotyrosine immunoblot of proteins from basal and insulin-stimulated 3T3-L1 adipocytes is shown (*right panel*). Insulin concentrations (in μU/ml) in the serum of the subject during the experimental procedure and in the culture medium of basal and insulin-stimulated 3T3-L1 adipocytes, respectively, are indicated. *B*: Adipocyte cell size analysis. Cell diameter of isolated subcutaneous (SC) and omental (O) adipocytes was determined with Scion software. A representative digital image (*left*) and quantitation of cell diameter from three experiments (*right*) are shown. *C*: GAPDH total protein content in human subcutaneous (SC) and omental (O) fat. Solubilized adipose tissue proteins were analyzed by immunoblotting with anti-GAPDH antibodies. A representative experiment (*left*) and the quantitation of results from four experimental subjects (*right*) are shown.**

30 min. For comparison, an immunoblot of tyrosine phosphoproteins from basal and insulin-stimulated 3T3-L1 adipocytes is also shown. Despite the fact that serum insulin concentrations achieved during the in vivo protocol were 20-fold lower than those in the culture medium of in vitro–stimulated adipocytes, insulin-induced tyrosine phosphorylation of insulin receptors and IRS proteins could be readily detected in fat in vivo. This demonstrates

TABLE 1
Characteristics of the experimental subjects

	Women	Men	*P*
n	4	3	—
Age (years)	57.3 ± 4.0	60.3 ± 10.7	0.78
BMI (kg/m^2)	25.3 ± 1.4	26.6 ± 1.3	0.50
BMI ≥25.0 kg/m^2 (*n*)	2	2	—
Body fat (%)	32.8 ± 4.1	22.6 ± 3.0	0.10
Fat mass (kg)	21.2 ± 4.1	15.8 ± 2.2	0.29
Lean mass (kg)	41.8 ± 2.2	54.4 ± 6.5	0.16
Total body water (kg)	30.6 ± 1.6	39.8 ± 4.8	0.16
Fasting glucose (mmol/l)	5.4 ± 0.2	5.1 ± 0.5	0.56
Fasting insulin (pmol/l)	45.6 ± 15.6	130.2 ± 34.8	0.08
K_{ITT}	4.1 ± 0.5	5.6 ± 1.0	0.21
Erythrocyte sedimentation rate (mm/h)	12.3 ± 0.9	12.0 ± 1.0	0.87
White blood cells (n/mm^3)	7,237 ± 230	8,000 ± 100	0.06
Fibrinogen (g/l)	2.94 ± 0.08	3.02 ± 0.13	0.71

Data are means ± SE.

the feasibility of the in vivo insulin stimulation protocol to investigate signaling reactions in human fat.

Characteristics of the experimental subjects. Subjects enrolled in the study had normal fasting glucose and insulin levels and were nonobese and not insulin resistant, as assessed by the ITT (Table 1). Body composition parameters and inflammation markers (i.e., erythrocyte sedimentation rate, white blood cell count, and fibrinogen) were within the normal range (Table 1). Paired abdominal subcutaneous and omental fat biopsies from the same individual were obtained at baseline and 6 and 30 min following insulin injection. Average protein yields from tissue samples were not different in subcutaneous and omental specimens (1.9 ± 0.3 vs. 1.5 ± 0.2 mg/100 mg tissue, $P = 0.209$). DNA recovery from fat biopsies varied widely and tended to be lower in subcutaneous fat, although this difference was not statistically significant (8.9 ± 2.8 vs. 17.2 ± 6.2 μg/100 mg tissue, $P = 0.113$). No differences were observed in GAPDH protein content in tissue protein extracts from the two fat depots ($P = 0.69$; Fig. 1*B*). Adipocytes isolated from subcutaneous and omental fat showed a similar cell diameter ($P = 0.60$; Fig. 1*C*).

Insulin receptor and IRS proteins. The levels of insulin receptor tyrosine phosphorylation and protein content were investigated in the subcutaneous and omental fat of the experimental subjects (Fig. 2). In omental fat, insulin induced a significant 2.5-fold increase in receptor tyrosine phosphorylation 6 min following its intravenous injection ($P < 0.05$ vs. basal), with lower levels after 30 min (Fig. 2*A*). In contrast, in the subcutaneous fat, basal receptor tyrosine phosphorylation was very low and showed slight increases following insulin stimulation at 6 min and 30 min (Fig. 2*A*), which, however, did not reach statistical significance ($P = 0.202$ and $P = 0.110$ vs. basal at 6 min and 30 min, respectively). Therefore, tyrosine phosphorylation of the insulin receptor was higher in omental than in subcutaneous fat both under basal conditions and following insulin stimulation ($P < 0.05$ vs. subcutaneous fat; Fig. 2*A*). The total amount of insulin receptor protein, determined by immunoblotting with anti–insulin receptor antibodies, was also higher in omental than subcutaneous fat ($P < 0.05$; Fig. 2*B*). When normalized per amount of insulin receptor protein (Fig. 2*C*), the levels of receptor phosphorylation were significantly augmented 6 min after insulin stimulation in omental fat ($P < 0.05$) but not in subcutaneous fat ($P = 0.247$), suggesting that the different temporal profile of insulin receptor activation in the two fat depots was independent of insulin receptor protein levels.

IRS-1, IRS-2, and the Shc proteins (i.e., p66^{Shc}, p52^{Shc}, and p46^{Shc}) represent the immediate substrates for the insulin receptor tyrosine kinase and act as scaffold molecules to mediate downstream insulin signaling. Similar to the insulin receptor protein, IRS-1 and IRS-2 protein levels, as determined by immunoblotting techniques, appeared to be increased 1.7- to 2.5-fold, respectively, in omental compared with subcutaneous fat samples; however, these differences reached statistical significance for IRS-2 only ($P = 0.078$ for IRS-1, $P < 0.05$ for IRS-2; Fig. 3*A* and *B*). In contrast, Shc protein content was similar in subcutaneous and omental fat ($P = 0.298$ for the p52^{Shc} protein; Fig. 3*C*). Both IRS-1 and IRS-2 proteins showed decreased electrophoretic mobility at 6 and 30 min compared with 0 min (Fig. 3*A* and *B*), likely a consequence of insulin-induced increase in their phosphoserine content. The levels of IRS-1, IRS-2, and Shc tyrosine phosphorylation, studied by sequential immunoprecipitation with the specific antibodies and immunoblotting with anti-phosphotyrosine antibodies, were below the detection threshold of the methods in various subjects (data not shown), and this did not allow us to perform thorough quantitative analyses. Upon insulin stimulation, the enzyme PI 3-kinase is activated following its binding to IRS proteins through its 85-kDa regulatory subunit. Therefore, the amount of p85 protein in IRS-1 immunoprecipitates was determined as a measure of the activation state of PI 3-kinase in human fat. In the omental fat, IRS-1–associated p85 was increased rapidly at 6 min following insulin stimulation, in contrast with the subcutaneous fat (Fig. 3*E*), mirroring the distinct temporal profile of insulin receptor phosphorylation in the two fat depots (Fig. 2*A*). The total amount of p85 was found to be increased approximately twofold in omental compared with subcutaneous protein tissue extracts ($P < 0.05$; Fig. 3*D*).

Akt and GSK-3. We next investigated the total protein and activation levels of Akt, a serine/threonine kinase important for insulin regulation of various metabolic responses in adipocytes (18). Since sequential phosphorylation of Akt on Ser-473 and Thr-308 is required for full activation of the enzyme, the levels of Akt phosphorylation on each amino acid residue were determined by immunoblotting with phospho-specific anti-Akt antibodies. Under basal conditions, phosphorylation of Akt on Ser-473 was

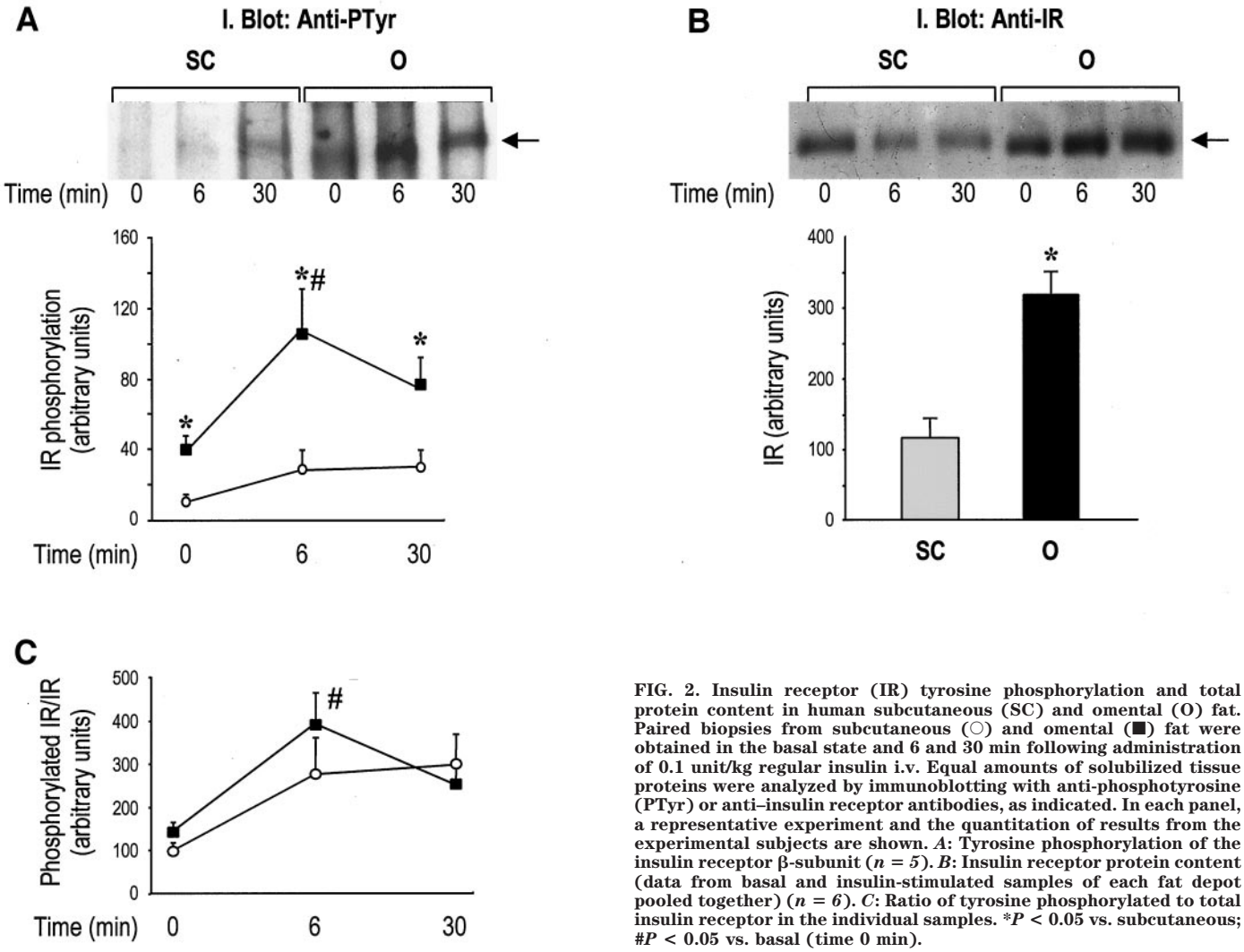

FIG. 2. Insulin receptor (IR) tyrosine phosphorylation and total protein content in human subcutaneous (SC) and omental (O) fat. Paired biopsies from subcutaneous (○) and omental (■) fat were obtained in the basal state and 6 and 30 min following administration of 0.1 unit/kg regular insulin i.v. Equal amounts of solubilized tissue proteins were analyzed by immunoblotting with anti-phosphotyrosine (PTyr) or anti–insulin receptor antibodies, as indicated. In each panel, a representative experiment and the quantitation of results from the experimental subjects are shown. *A*: Tyrosine phosphorylation of the insulin receptor β-subunit ($n = 5$). *B*: Insulin receptor protein content (data from basal and insulin-stimulated samples of each fat depot pooled together) ($n = 6$). *C*: Ratio of tyrosine phosphorylated to total insulin receptor in the individual samples. *$P < 0.05$ vs. subcutaneous; #$P < 0.05$ vs. basal (time 0 min).

not significantly different in omental and subcutaneous fat ($P = 0.16$; Fig. 4*A*). Upon insulin stimulation, Akt phosphorylation on Ser-473 was increased several-fold in both subcutaneous and omental fat (Fig. 4*A*). However, in omental fat, Akt phosphorylation on Ser-473 was highest 6 min following insulin administration ($P < 0.05$ vs. basal) and remained elevated to similar levels after 30 min; by contrast, in subcutaneous fat, Akt phosphorylation on Ser-473 was very low 6 min following insulin injection and increased significantly only after 30 min ($P < 0.05$ vs. basal; Fig. 4*A*). Akt phosphorylation on Thr-308, which was not different in the two fat depots at baseline ($P = 0.30$), also showed earlier and more intense phosphorylation levels in omental compared with subcutaneous fat upon insulin stimulation ($P < 0.05$, omental versus subcutaneous fat at 6 min; Fig. 4*B*). Akt protein levels were similar in omental and subcutaneous fat tissue specimens ($P = 0.25$; Fig. 4*C*).

To assess whether the observed temporal profiles and levels of Akt phosphorylation in the omental and subcutaneous fat depots were paralleled by differences in Akt activity, the phosphorylation of the endogenous Akt substrate GSK-3 was next examined by immunoblotting with phospho-specific anti–GSK-3 antibodies. GSK-3 is directly phosphorylated by Akt on Ser-9 (α-isoform) or Ser-21 (β-isoform) and is inactivated following its phosphorylation. Basal GSK-3 phosphorylation was not significantly different in omental and subcutaneous fat ($P = 0.20$ and $P = 0.46$ for GSK-3α and GSK-3β, respectively; Fig. 5*A*). Insulin injection resulted in increased phosphorylation of both GSK-3α and GSK-3β by several-fold in omental fat, which was statistically significant after 6 min of stimulation ($P < 0.05$ vs. basal for both isoforms; Fig. 5*A*). By contrast, changes in GSK-3 phosphorylation induced by insulin administration were minimal and statistically nonsignificant in subcutaneous fat ($P = 0.34$ and $P = 0.46$ for GSK-3α and GSK-3β, respectively; Fig. 5*A*). Therefore, the levels of insulin-stimulated GSK-3α and GSK-3β phosphorylation were significantly higher in omental than in subcutaneous fat after both 6 and 30 min of stimulation ($P < 0.05$; Fig. 5*A*). The observed changes in GSK-3 phosphorylation were associated with increased expression levels of GSK-3α and GSK-3β proteins in omental fat, as determined by immunoblotting with anti–GSK-3 antibody ($P < 0.05$ vs. subcutaneous fat for both GSK-3α and GSK-3β; Fig. 5*B*). When normalized per amount of GSK-3 protein (Fig. 5*C*), the levels of GSK-3α phosphorylation were augmented upon insulin stimulation significantly in omental fat ($P < 0.05$ vs. basal) but not in subcutaneous fat ($P = 0.18$ vs. basal), indicating that enhancement of GSK-3 phosphorylation by insulin was independent of GSK-3 protein levels. Therefore, both Akt and GSK-3 show more rapid and pronounced responses to insulin stimulation in omental than in subcutaneous adipose tissue.

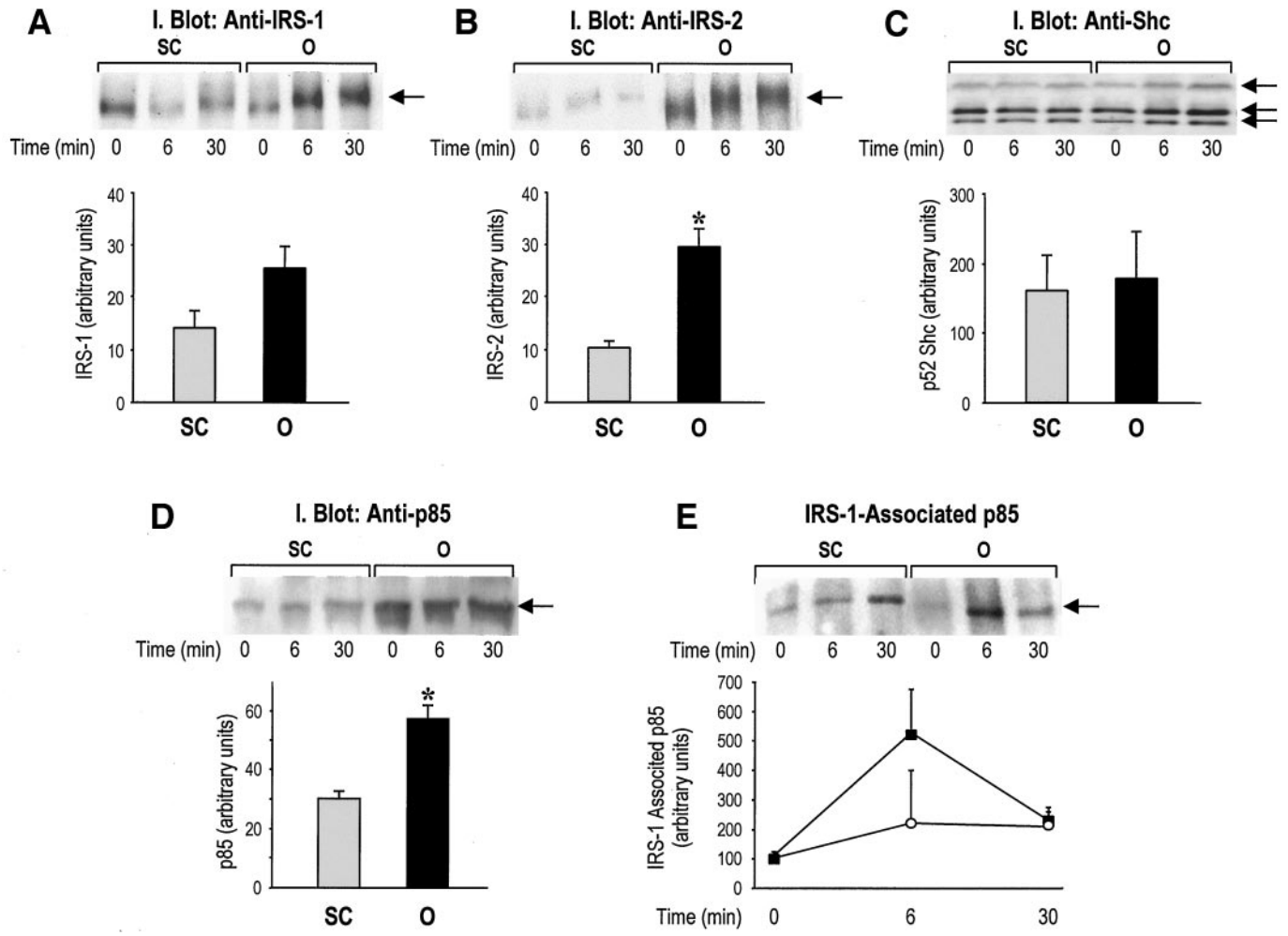

FIG. 3. IRS-1, IRS-2, p85, and Shc protein content in human subcutaneous (SC) and omental (O) fat. Paired biopsies from subcutaneous (○) and omental (■) fat were obtained in the basal state and 6 and 30 min following administration of 0.1 unit/kg regular insulin i.v. Equal amounts of solubilized tissue proteins were analyzed by immunoblotting with anti–IRS-1, anti–IRS-2, anti-p85, or anti-Shc antibodies, respectively. In each panel, a representative experiment and the quantitation of results from all experimental subjects are shown. *A*: IRS-1 protein content ($n = 6$). *B*: IRS-2 protein content ($n = 7$). *C*: Shc protein content and quantitation of the $p52^{Shc}$ isoform ($n = 6$). *D*: p85 protein content ($n = 4$). To quantitate the total content of these proteins, data from basal and insulin-stimulated samples of each fat depot were pooled. *E*: IRS-1–associated p85. Equal amounts of solubilized tissue proteins were immunoprecipitated with anti–IRS-1 antibodies and analyzed by immunoblotting with anti-p85 antibodies ($n = 4$). *$P < 0.05$ vs. subcutaneous.

ERK-1/2. Activation of ERK by insulin was investigated in human visceral and subcutaneous fat because this kinase is involved in insulin-mediated regulation of adipocyte transcription factors and adipose tissue development. Under basal conditions, phosphorylation of ERK-1 and ERK-2 was not different in the two adipose tissues ($P = 0.12$ and $P = 0.54$ for ERK-1 and ERK-2, respectively; Fig. 6*A*). Insulin injection induced marked and significant increases in ERK-1 and ERK-2 phosphorylation in omental fat after 6 min of stimulation ($P < 0.05$ vs. basal; Fig. 6*A*). In contrast, phosphorylation of ERK-1 and ERK-2 was significantly increased by insulin after 30 min in subcutaneous fat (Fig. 6*A*). Protein content of both ERK-1 and ERK-2 was found to be approximately threefold higher in omental than in subcutaneous fat samples ($P < 0.05$ for both isoforms; Fig. 6*B*). When normalized per amount of ERK protein, the levels of ERK-1 phosphorylation were found to be significantly stimulated by insulin after 6 min in omental fat ($P < 0.05$ vs. basal) and after 30 min in subcutaneous fat ($P < 0.05$ vs. basal) (Fig. 6*C*), indicating that the distinct temporal profile of ERK activation in the two tissues was independent of differences in ERK protein levels. Therefore, ERK phosphorylation was induced by insulin earlier in omental than in subcutaneous fat, analogous with Akt and GSK-3.

To investigate whether other signaling proteins in the Ras-MAP kinase pathway were differently expressed in omental versus subcutaneous fat, the total protein content of MEK (MAP kinase kinase) and Elk-1 (an ERK-dependent transcription factor) was determined. MEK protein levels were found to be increased in the omental compared with the subcutaneous fat (268.1 ± 39.8 vs. 127.2 ± 11.4, $P < 0.05$), whereas no differences could be observed in Elk-1 protein levels in the two fat depots (89.8 ± 22.8 vs. 80.3 ± 18.4, $P = 0.60$).

DISCUSSION

In this study, we analyzed the insulin signaling system in abdominal visceral (omental) and subcutaneous fat depots of nonobese, nondiabetic humans. The total protein levels of multiple insulin signaling intermediates, including the insulin receptor, IRS-2, p85, GSK-3α, GSK-3β, MEK, ERK-1, and ERK-2, were found to be higher in omental than

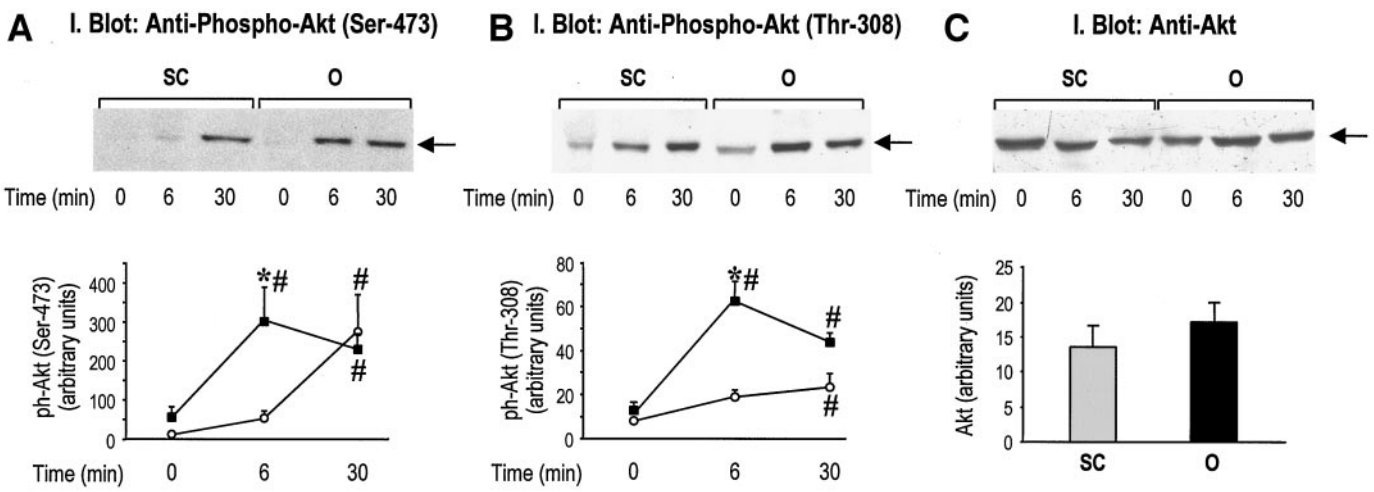

FIG. 4. Akt phosphorylation and total Akt protein content in human subcutaneous (SC) and omental (O) fat. Solubilized proteins from subcutaneous (○) and omental (■) fat depots were resolved by 10% SDS-PAGE and analyzed by immunoblotting with anti–phospho-Akt (Ser-473), anti–phospho-Akt (Thr-308), or anti-Akt antibodies, as described in RESEARCH DESIGN AND METHODS. *A* and *B*: Representative immunoblots and the quantitation of Akt phosphorylation on Ser-473 and Thr-308, respectively ($n = 6$). *C*: Total Akt protein content ($n = 6$) (values of basal and insulin-stimulated samples pooled together). *$P < 0.05$ vs. SC; #$P < 0.05$ vs. basal (time 0 min).

subcutaneous fat. Importantly, when activation of the insulin signaling cascade was studied following administration of an intravenous bolus insulin injection, greater and earlier activation levels of the insulin receptor, Akt, GSK-3, and ERK-1/2 were found in omental than in subcutaneous adipose tissue. To our knowledge, this is the first study in which insulin signaling was investigated in vivo in humans in fat from two distinct body regions.

The greater content of signaling proteins in omental compared with subcutaneous fat in this study could potentially derive from a higher number of cells per gram of tissue specimen analyzed. Visceral adipocytes have been

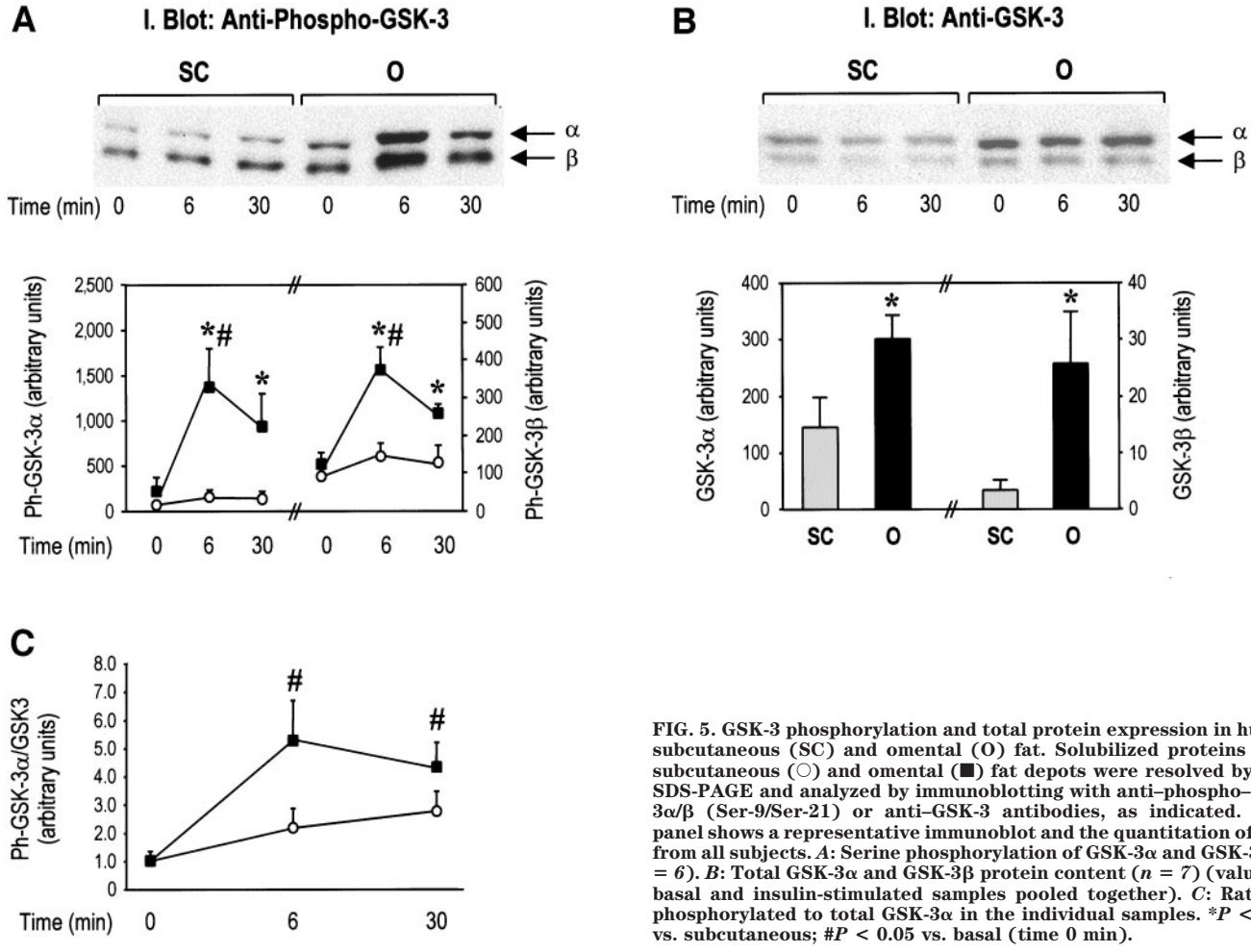

FIG. 5. GSK-3 phosphorylation and total protein expression in human subcutaneous (SC) and omental (O) fat. Solubilized proteins from subcutaneous (○) and omental (■) fat depots were resolved by 10% SDS-PAGE and analyzed by immunoblotting with anti–phospho–GSK-3α/β (Ser-9/Ser-21) or anti–GSK-3 antibodies, as indicated. Each panel shows a representative immunoblot and the quantitation of data from all subjects. *A*: Serine phosphorylation of GSK-3α and GSK-3β ($n = 6$). *B*: Total GSK-3α and GSK-3β protein content ($n = 7$) (values of basal and insulin-stimulated samples pooled together). *C*: Ratio of phosphorylated to total GSK-3α in the individual samples. *$P < 0.05$ vs. subcutaneous; #$P < 0.05$ vs. basal (time 0 min).

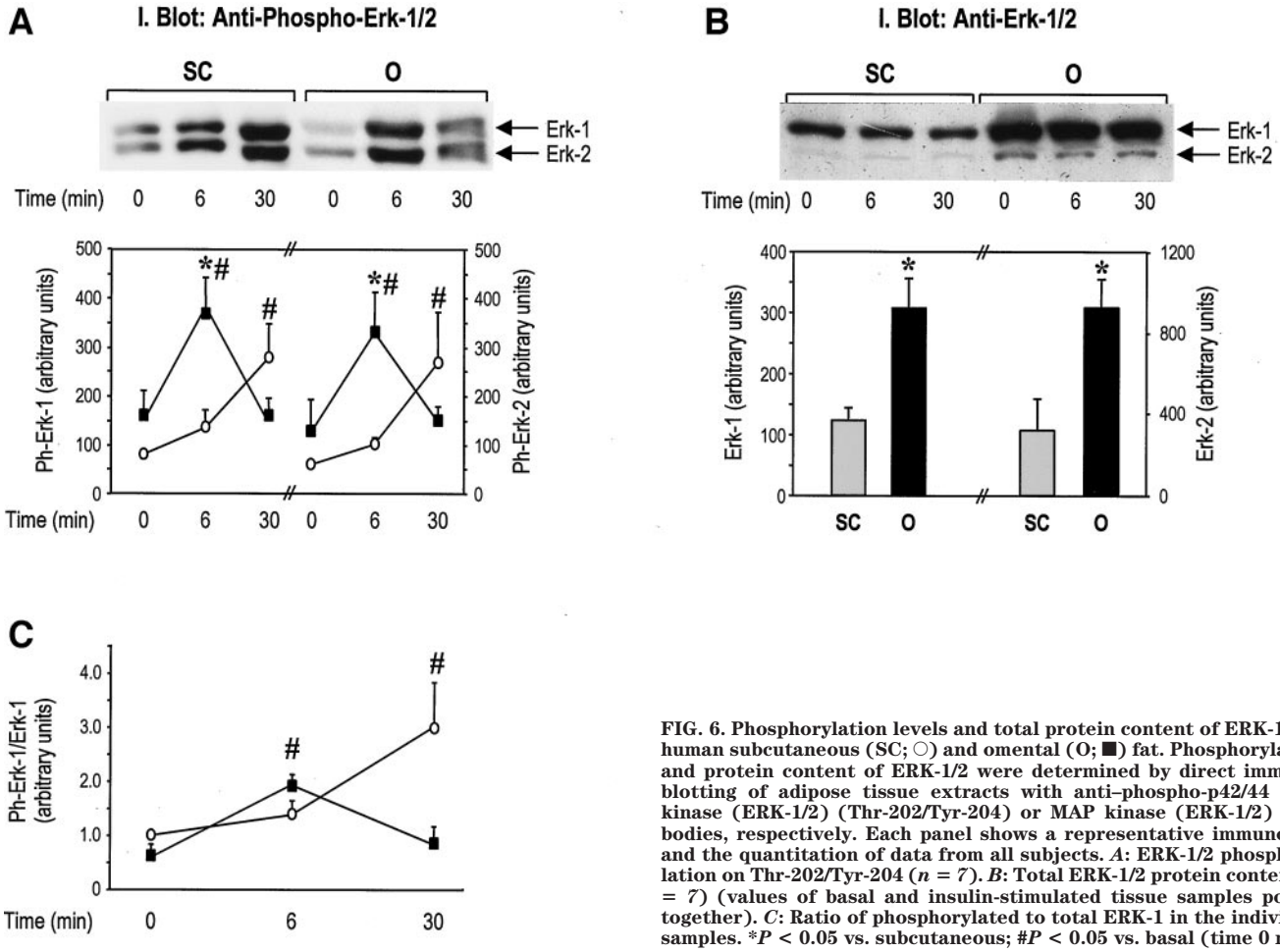

FIG. 6. Phosphorylation levels and total protein content of ERK-1/2 in human subcutaneous (SC; ○) and omental (O; ■) fat. Phosphorylation and protein content of ERK-1/2 were determined by direct immunoblotting of adipose tissue extracts with anti–phospho-p42/44 MAP kinase (ERK-1/2) (Thr-202/Tyr-204) or MAP kinase (ERK-1/2) antibodies, respectively. Each panel shows a representative immunoblot and the quantitation of data from all subjects. *A*: ERK-1/2 phosphorylation on Thr-202/Tyr-204 ($n = 7$). *B*: Total ERK-1/2 protein content ($n = 7$) (values of basal and insulin-stimulated tissue samples pooled together). *C*: Ratio of phosphorylated to total ERK-1 in the individual samples. *$P < 0.05$ vs. subcutaneous; #$P < 0.05$ vs. basal (time 0 min).

suggested to be smaller than subcutaneous cells (24). However, not all studies have confirmed these findings (19), and no differences in visceral versus subcutaneous adipocyte cell size or tissue DNA yield were observed in this study. It should also be noted that omental samples were characterized by higher protein content of some but not all signaling proteins because expression levels of IRS-1, Shc, Elk, and Akt did not significantly differ in the two fat depots. In addition, the tissue content of GAPDH, which is commonly considered a housekeeping protein, was not different in subcutaneous and omental biopsies. Similar results have been recently reported by Lundgren et al. (24), who showed equal cellular content of IRS-1 and Akt in adipocytes isolated from omental and subcutaneous fat. Altogether, these results suggest that the increased expression levels of the insulin receptor, IRS-2, p85, GSK-3, MEK, and ERK in omental fat could represent, at least in part, intrinsic characteristics of the adipocytes from this fat depot. The increase in protein levels of some of these signaling molecules could potentially result in apparently enhanced phosphorylation levels when determined by direct immunoblotting. However, in the case of GSK-3, the increase in phosphorylation persisted even after normalization per amount of total GSK-3 protein (Fig. 5*C*); in the case of the insulin receptor (Fig. 2*C*) and ERK-1/2 (Fig. 6*C*), the phosphorylation kinetics were clearly different in the two fat depots, and this is unlikely to be explained on the basis of changes in total protein levels of these signaling molecules. In contrast to subcutaneous fat, the responses of Akt/GSK-3 and ERK-1/2 to insulin were more rapid but also somewhat more transient in omental fat because activation levels in this depot were higher at 6 than at 30 min. The temporal profile of activation of these signaling proteins parallels the activation profile of the insulin receptor tyrosine kinase (Fig. 2*A*). Since both total phosphotyrosine phosphatase and protein tyrosine phosphatase 1B activities were found to be increased in omental compared with subcutaneous fat in humans (25), the more transient activation patterns of the insulin receptor and downstream signaling molecules in response to acute insulin stimulation in visceral fat could be potentially explained by more efficient mechanisms of receptor dephosphorylation and signal cessation.

Visceral fat may display an increased metabolic activity in regard to insulin action. In a recent study, Virtanen et al. (26) analyzed fat glucose uptake and blood perfusion in humans in vivo using positron emission tomography combined with magnetic resonance imaging and isotope labeling. Insulin-stimulated glucose uptake per surface area was found to be higher in visceral than subcutaneous fat in both normal-weight and obese subjects. Importantly, no significant differences in tissue blood flow between the two fat depots were reported in that study, suggesting that the greater response to insulin in visceral fat was not the consequence of more efficient hormone delivery to the cell site of action but rather reflected specific characteristics of the adipocytes from this fat depot. Indeed, insulin-stimulated glucose uptake was found to be approximately twofold higher in isolated human omental compared with subcutaneous adipocytes (24). In that study, higher ex-

pression levels of GLUT4 were also demonstrated in omental adipocytes. The greater ability of insulin to promote glucose uptake in visceral compared with subcutaneous fat could also be potentially explained by higher levels of insulin-stimulated activation of insulin receptors and Akt in the former fat depot, as shown in this study.

Inhibition of lipolysis by insulin has been shown to require Akt-dependent phosphorylation and activation of the phosphodiesterase 3B, resulting in cAMP hydrolysis and interference with cathecolamine-mediated stimulation of protein kinase A and hormone-sensitive lipase activities (27). The increased Akt activation found in visceral fat in this study does not appear to explain previous observations on the lower ability of insulin to inhibit catecholamine-induced lipolysis in visceral compared with subcutaneous fat tissues (28) and isolated adipocytes (29). It is possible that lower activity and/or expression levels of phosphodiesterase 3B in visceral than in subcutaneous fat may explain these observations; this possibility is currently being addressed in our laboratory. The differences in insulin signaling between visceral and subcutaneous fat observed in this study are also apparently in contrast with the results of Zierath et al. (19), who reported reduced levels of insulin-stimulated tyrosine phosphorylation of insulin receptors and IRS-1 and PI 3-kinase activity in visceral compared with subcutaneous adipocytes. However, it should be noted that the subjects enrolled in that study had a wide range of BMI values and included markedly obese individuals (19). Since obesity has been shown to be associated with impaired insulin-stimulated IRS-1 tyrosine phosphorylation in adipocytes (30), the inclusion of both nonobese and obese subjects in that analysis may have masked physiological differences in insulin signaling between the two fat depots. Additionally, it must be pointed out that measurements of lipolysis and insulin signaling in those previous studies were carried out on adipocytes isolated from their anatomical location and subsequently analyzed in vitro in the presence of pharmacological doses of hormonal agonists. In contrast, activation of insulin signaling in the present study occurred entirely in vivo following achievement of serum insulin concentrations close to the physiological range (Fig. 1). Under these experimental conditions, the biochemical reactions occurring in the adipose tissue are likely to be influenced by a complex network of signaling inputs generated by the tissue micro-environment. Indeed, nonadipocyte cellular components of the fat possessing depot-specific features may have contributed to the observed differences. For example, visceral fat contains higher plasminogen activator inhibitor 1 (PAI-1) levels than subcutaneous fat, and this is related to its greater enrichment in stromal cells, the principal source of PAI-1 in adipose tissue (31). PAI-1 has been shown to modulate insulin signaling (32). Higher proportions of endothelial cells and macrophages have also been described in visceral compared with subcutaneous fat (33–35). Cytokines released by these cell types may also modulate adipocyte signaling responses to insulin, as it has been shown for endothelin-1 (36,37). It may be difficult to appreciate the effects of all of these factors on insulin signaling when studies on adipocytes are conducted in vitro.

The physiological significance of the different magnitude and temporal profile of insulin-stimulated ERK activation in visceral and subcutaneous fat is still largely unknown. In particular, it is not clear whether earlier and more transient ERK-1/2 activation in the visceral fat, as opposed to the gradual and more sustained activation of this kinase in the subcutaneous fat, may be coupled to distinct cellular effects of insulin in the two tissue depots. However, it should be recognized that a different temporal profile of receptor and postreceptor signaling reactions may lead to distinct insulin bio-effects (38,39). ERK-1 plays a critical role in adipocyte growth and differentiation, as recently shown by studies in knock-out mice lacking ERK-1, which have decreased adiposity and fewer adipocytes than wild-type animals (40). Since subcutaneous and omental preadipocytes in culture exhibit different proliferation and differentiation capacities (34,41), it is possible that this difference may be related to distinct ERK activation kinetics in the two cell populations. In addition, ERK-1/2 has been shown to modulate expression and phosphorylation of PPAR-γ (42); thus, it will be important to investigate further the relationship between ERK activation and regulation of PPAR-γ (and potentially other adipose tissue transcription factors) in distinct fat depots.

In conclusion, we have analyzed and compared insulin signaling reactions in human omental and subcutaneous fat in vivo. Omental fat is characterized by higher expression levels of specific signaling proteins and more pronounced and/or earlier activation of the Akt/GSK-3 and ERK signaling pathways in response to insulin administration. In future studies, it will be interesting to use this in vivo analysis of insulin signaling in human fat for investigation of depot-specific abnormalities in obesity and other insulin-resistant states.

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