

# Impaired Activation of Protein Kinase C- $\zeta$ by Insulin and Phosphatidylinositol-3,4,5-( $\text{PO}_4$ ) $_3$ in Cultured Preadipocyte-Derived Adipocytes and Myotubes of Obese Subjects

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**Insulin resistance in obesity is partly due to diminished glucose transport in myocytes and adipocytes, but underlying mechanisms are uncertain. Insulin-stimulated glucose transport requires activation of phosphatidylinositol (PI) 3-kinase (3K), operating downstream of insulin receptor substrate-1. PI3K stimulates glucose transport through increases in PI-3,4,5-( $\text{PO}_4$ ) $_3$  ( $\text{PIP}_3$ ), which activates atypical protein kinase C (aPKC) and protein kinase B (PKB/Akt). However, previous studies suggest that activation of aPKC, but not PKB, is impaired in intact muscles and cultured myocytes of obese subjects. Presently, we examined insulin activation of glucose transport and signaling factors in cultured adipocytes derived from preadipocytes harvested during elective liposuc-**

**tion in lean and obese women. Relative to adipocytes of lean women, insulin-stimulated [ $^3\text{H}$ ]2-deoxyglucose uptake and activation of insulin receptor substrate-1/PI3K and aPKCs, but not PKB, were diminished in adipocytes of obese women. Additionally, the direct activation of aPKCs by  $\text{PIP}_3$  *in vitro* was diminished in aPKCs isolated from adipocytes of obese women. Similar impairment in aPKC activation by  $\text{PIP}_3$  was observed in cultured myocytes of obese glucose-intolerant subjects. These findings suggest the presence of defects in PI3K and aPKC activation that persist in cultured cells and limit insulin-stimulated glucose transport in adipocytes and myocytes of obese subjects. (*J Clin Endocrinol Metab* 89: 3994–3998, 2004)**

INSULIN RESISTANCE IS frequently observed in obesity, an important component of the “metabolic syndrome” and a common forerunner to type 2 diabetes. Such insulin resistance in these interrelated disorders is partly due to diminished activation of glucose transport in skeletal muscle and adipocytes, but underlying mechanisms are obscure. Insulin-stimulated glucose transport, which is known to be diminished in freshly isolated adipocytes of obese subjects (1) and cultured myotubes of obese glucose-intolerant subjects (2), requires the activation of phosphatidylinositol (PI) 3-kinase (3K), operating downstream of insulin receptor substrates, including insulin receptor substrate-1 (IRS-1). PI3K in turn stimulates glucose transport through increases in levels of PI-3,4,5-( $\text{PO}_4$ ) $_3$  ( $\text{PIP}_3$ ) and subsequent activation of atypical protein kinase C (aPKC) (3–6) and protein kinase B (PKB/Akt) (7–10).

Previous studies in type 2 diabetic subjects have revealed defects in insulin activation of: 1) IRS-1-dependent PI3K in adipocytes (11) and skeletal muscle (12–15); 2) PKB activation in adipocytes (11) and in some (13), but not all (14, 15), studies of muscle; and 3) aPKC activation in muscle (15, 16). In obese glucose-intolerant humans, defects in insulin activation of

aPKCs, but not IRS-1, have been observed in biopsies of skeletal muscle taken during hyperinsulinemic/euglycemic clamp studies (15) and in cultured myotubes (2). Similarly, in hyperinsulinemic/euglycemic clamp studies of obese glucose-tolerant humans, a defect in insulin activation of aPKCs, but not PKB, was observed in skeletal muscle (Ref. 16 and unpublished observations), and a defect in IRS-1-dependent PI3K activation has been observed in isolated muscle strips (17).

In contrast to muscle, there is little or no available information on the activation of the above-described insulin-sensitive signaling factors that operate downstream of PI3K in adipocytes of obese humans. Such information is important, because 1) adipocytes and myocytes may have common insulin signaling defects, 2) defects in adipocytes, via hormonal or metabolic factors, may contribute to defects in muscle, 3) adipocytes may contribute importantly to total body glucose metabolism in obese subjects, and 4) defects in insulin-stimulated glucose transport in isolated adipocytes *in vitro* correlate well with, and thus appear to be generally reflective of, defects in whole-body insulin-stimulated glucose disposal *in vivo* (1).

Presently, we examined insulin effects on glucose transport and activation of the above-described signaling factors in cultures of adipocytes derived from preadipocytes harvested during elective liposuction in lean and obese women. Relative to adipocytes derived from lean subjects, insulin-stimulated glucose transport and activation of IRS-1-dependent PI3K and aPKC, but not PKB, were diminished in adipocytes derived

Abbreviations: aPKC, Atypical protein kinase C; BMI, body mass index; IRS-1, insulin receptor substrate-1; 3K, 3-kinase; PI, phosphatidylinositol;  $\text{PIP}_3$ , PI-3,4,5-( $\text{PO}_4$ ) $_3$ ; PKB/Akt, protein kinase B.

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from obese women. In addition to the impairment in aPKC activation by insulin in intact cells, the direct activation by PIP<sub>3</sub> *in vitro* was diminished in aPKCs isolated from adipocytes of obese women. A similar impairment in responsiveness of aPKCs to PIP<sub>3</sub> was observed in cultured myotubes obtained from muscles of obese glucose-intolerant subjects. Our findings suggest the presence of defects in IRS-1-dependent PI3K and aPKC activation that limit insulin-stimulated glucose transport in adipocytes of obese women.

## Subjects and Methods

### Studies in adipocytes

As described (18), sc adipose tissue from abdominal and thigh areas was obtained by Zen-Bio Inc. (Research Triangle Park, NC) from lean [body mass index (BMI), 21 ± 1 kg/m<sup>2</sup>; age, 43 ± 3 yr] and obese (BMI, 34 ± 2; age, 45 ± 4 yr), otherwise healthy, nondiabetic women undergoing cosmetic elective liposuction procedures. Institutional review board approval and informed consent for use of the adipose tissue were obtained from the patients by Zen-Bio Inc. All women in these studies had normal fasting blood glucose values, *i.e.* less than 6.1 mM, and, as per exclusion criteria, had no significant medical problems and were not taking any medications. No other clinical information was available on these subjects, in conformity with the design of the institutional review board-approved protocol, which limited gathered information only to height, weight, gender, ethnicity, and location of the adipose tissue and precluded the recording of other clinical information, thus maintaining strict patient confidentiality and ensuring full compliance with the Health Insurance Portability and Accountability Act. In effect, tissue that would otherwise be discarded after the surgical procedure was voluntarily donated by the patient to Zen-Bio Inc.; neither physician nor patient received any compensation for this donation.

After digestion of adipose tissue with collagenase, preadipocytes were harvested, passaged two times, and then differentiated to mature lipid-laden adipocytes by incubation in DMEM/F-10 medium (1:1) containing 3% fetal bovine serum (Sigma, St. Louis, MO), insulin (Sigma), isobutyl methylxanthine (Sigma), and dexamethasone (Sigma).

Fully differentiated adipocytes were initially incubated for 48 h in DMEM/F-10 medium containing 3% fetal bovine serum. The medium was then removed, and cells were incubated for 3–4 h in serum-free DMEM/F-10 medium containing 4% BSA (Sigma). The adipocytes were then equilibrated for 30 min in glucose-free Krebs Ringer phosphate medium containing 1% BSA and finally incubated with or without insulin, as described in the text. After incubation, as described (15, 18, 19), cell lysates were examined for immunoprecipitable IRS-1-dependent PI3K or aPKC activity, PKB phosphorylation (*i.e.* phosphorylation of serine-473), uptake of [<sup>3</sup>H]-deoxyglucose, and levels of immunoreactive signaling factors.

Antisera used in Western analyses and/or immunoprecipitations included: rabbit polyclonal anti-IRS-1 antiserum [Upstate Biotechnologies, Inc. (UBI), Waltham, MA]; rabbit polyclonal anti-C-terminal PKC- $\zeta$ / $\lambda$ / $\iota$  antiserum (Santa Cruz Biotechnologies, Santa Cruz, CA) [note:

aPKCs  $\zeta$ ,  $\lambda$ , and  $\iota$  have nearly identical C termini that are recognized by this antiserum; however, human adipocytes only contain PKC- $\zeta$  (18)]; sheep polyclonal anti-PKB $\alpha$  antiserum (UBI); rabbit polyclonal antiphosphoserine-473-PKB antiserum (Cell Signaling Technology Inc., Beverly, MA); and mouse monoclonal anti-Glut4 antibodies (Biogenesis Inc., Kingston, NH).

Note that these preadipocyte-derived adipocytes contain Glut4 glucose transporters, and PKC- $\zeta$ , the major aPKC in these cells, is required for insulin-stimulated glucose transport (18). Also note that there were no apparent differences in histological appearances, including cell size and lipid content, as per oil red staining, of adipocytes derived from lean and obese women.

All *P* values in Figures 1–5 were determined by a one-way ANOVA followed by the least significant difference multiple comparison method.

### Studies in myotubes

Details of these studies and clinical characteristics of these subjects were described previously (2). In brief, myotubes were differentiated from muscle cells obtained from muscle biopsies taken from age-matched, lean (BMI < 25) healthy control and obese (BMI > 30) glucose-intolerant (blood glucose levels >6.1 but <6.9 mM fasting, or >7.8 but <11.1 mM 2 h after a standard 75-g oral glucose load) subjects. Note that the subjects used for myotubes studies were entirely different from subjects used in adipocyte studies. Also note that the myotube studies involved obese subjects who had glucose intolerance, whereas the adipocyte studies involved simply obese subjects.

The myotubes of obese glucose-intolerant subjects were originally found to have defects in insulin-stimulated glucose transport and activation of IRS-2-dependent PI3K and aPKCs, but not PKB (2). Presently, we examined aPKC activation by PIP<sub>3</sub> in frozen lysates from basal and insulin-treated myotubes obtained from lean control and obese glucose-intolerant subjects.

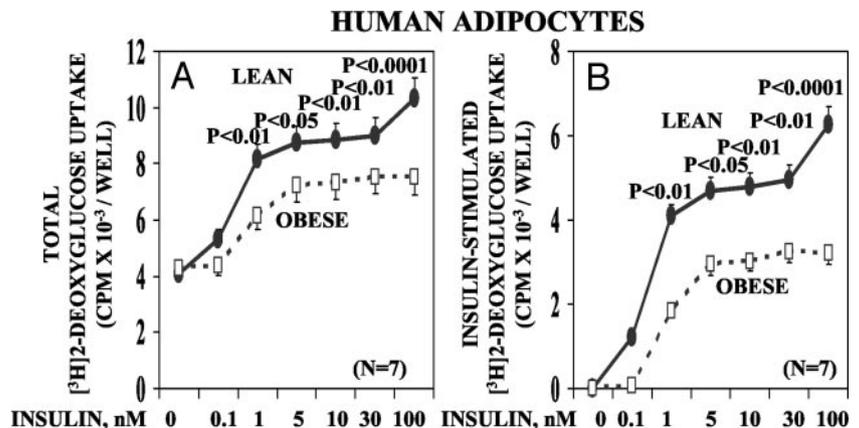
## Results

### Studies in adipocytes

**[<sup>3</sup>H]-2-Deoxyglucose uptake.** Insulin provoked 2- to 3-fold increases in [<sup>3</sup>H]-2-deoxyglucose uptake in adipocytes derived from lean subjects (Fig. 1). Relative to results seen in adipocytes of lean subjects, insulin-stimulated, but not basal, [<sup>3</sup>H]-2-deoxyglucose uptake was diminished in adipocytes derived from obese women (Fig. 1).

**IRS-1-dependent PI3K activation.** In conjunction with decreases in [<sup>3</sup>H]-2-deoxyglucose uptake, and relative to findings seen in adipocytes derived from lean subjects, the activation of IRS-1-dependent PI3K by insulin was markedly diminished in adipocytes of obese women (Fig. 2). However, the degree of diminution in IRS-1-dependent PI3K activation was much greater than the degree of diminution in insulin-

FIG. 1. Dose-related effects of insulin on [<sup>3</sup>H]-2-deoxyglucose uptake in adipocytes derived from lean or obese women. Uptake was determined over a 5-min period after a 30-min treatment with insulin. Total uptake and insulin-stimulated uptake are shown at left and right, respectively. Values are mean ± SE of the number of determinations (n) shown in parentheses. *P* values reflect comparison of uptake values at indicated insulin concentrations.



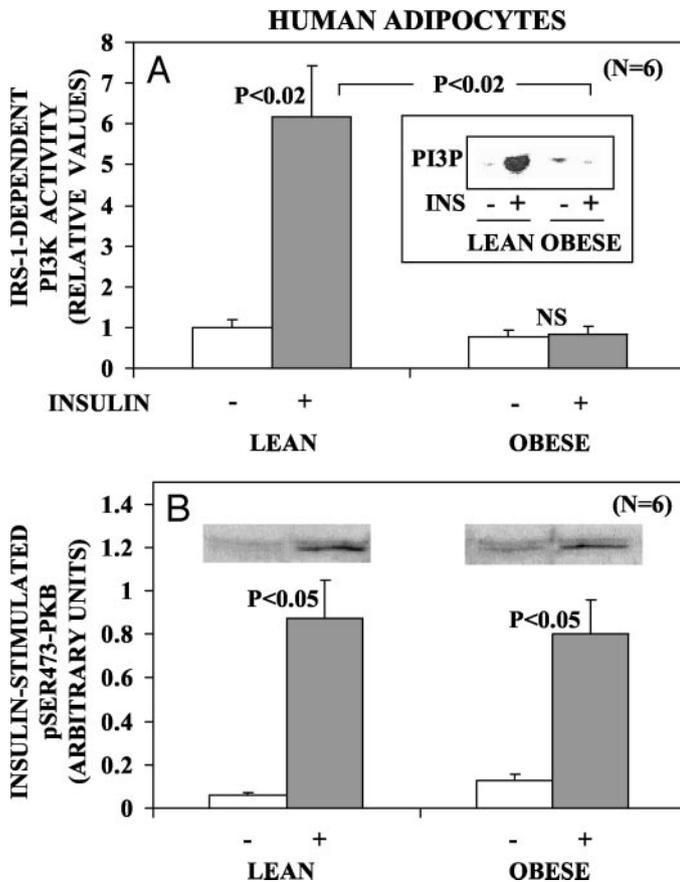


FIG. 2. Effects of insulin on IRS-1-dependent PI3K activity (A) and PKB phosphorylation (ser473) (B) in adipocytes derived from lean and obese women. Cells were treated for 15 min with or without 100 nM insulin. Values are mean  $\pm$  SE of the number of determinations (n) shown in parentheses. PI3K values from each of the four groups were normalized to the mean lean control/unstimulated value, set at unity, observed within the same autoradiogram. Phospho-PKB values were determined by scanning laser densitometry of x-ray films of basal and insulin-stimulated samples of both lean and obese subjects within the same immunoblot; note that these values are in actual arbitrary scan units. Insets in A and B portray representative autoradiograms and immunoblots, respectively.

stimulated [ $^3$ H]2-deoxyglucose uptake in adipocytes derived from obese women.

**PKB phosphorylation.** In contrast to the poor activation of IRS-1-dependent PI3K, insulin-stimulated phosphorylation of serine-473 in PKB (presumably reflecting activation) was not significantly different in adipocytes derived from lean and obese women (Fig. 2).

**PKC- $\zeta$  activation.** In contrast to the apparently normal phosphorylation/activation of PKB, but in keeping with the observed defect in activation of IRS-1-dependent PI3K, the activation of PKC- $\zeta$  by insulin was diminished in adipocytes derived from obese women (Fig. 3). This defect in insulin-stimulated PKC- $\zeta$  activation was similar in magnitude to the defect in insulin-stimulated [ $^3$ H]2-deoxyglucose uptake observed in adipocytes derived from obese women.

We have previously found that: 1) direct addition of PIP $_3$ , the lipid product of PI3K, to PKC- $\zeta$ / $\lambda$  immunoprecipitates obtained from adipocyte and/or muscle lysates taken from

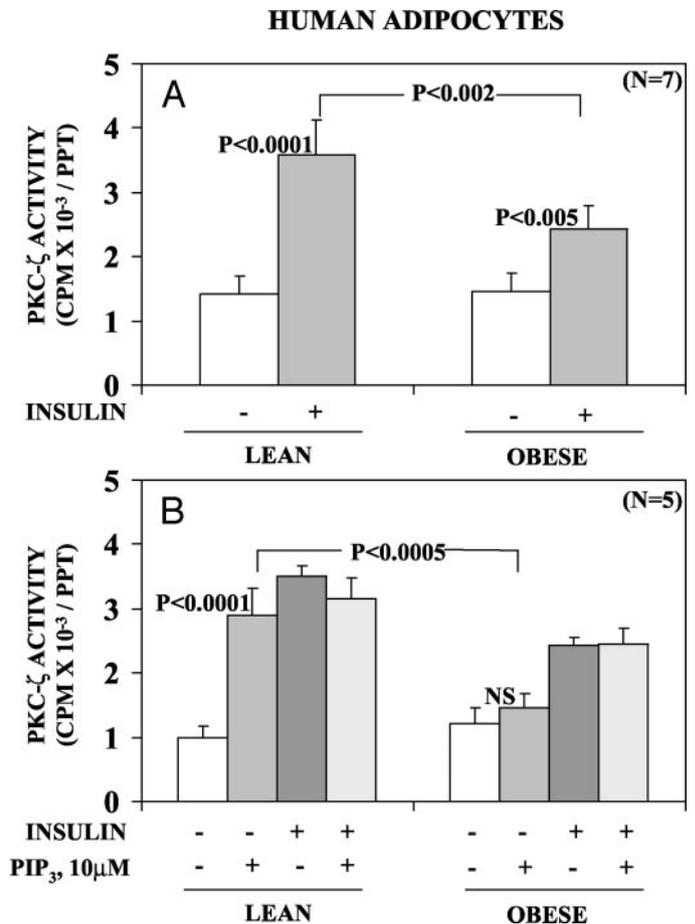


FIG. 3. Effects of insulin (A) and PIP $_3$  (B) on PKC- $\zeta$  activity in adipocytes derived from lean and obese women. A and B, Cells were treated for 15 min with or without 100 nM insulin. A, Lysates were subjected to aPKC assay directly. B, Immunoprecipitates were prepared from lysates of adipocytes treated with or without insulin, as indicated, and then assayed in the presence or absence of 10  $\mu$ M PIP $_3$ . Values are mean  $\pm$  SE of the number of determinations (n) shown in parentheses.

basal control, but not insulin-stimulated, rats (4), monkeys (19), and humans (15) provokes insulin-like increases in PKC- $\zeta$  enzyme activity; and 2) the activation of PKC- $\zeta$  is diminished, not only by insulin in intact muscles, but also by PIP $_3$  in immunoprecipitates prepared from muscles of type 2 diabetic monkeys (19) and humans (15) and obese women (Sagun, M. P., M. L. Standaert, A. Miuna, G. Bandyopadhyay, unpublished observations). Therefore, it was of great interest to find that, unlike results seen with PKC- $\zeta$  immunoprecipitates prepared from adipocytes of lean women, who responded to PIP $_3$  with insulin-like increases in PKC- $\zeta$  activity, there was poor activation of PKC- $\zeta$  by PIP $_3$  in PKC- $\zeta$  immunoprecipitates prepared from adipocytes derived from obese women (Fig. 3).

**Immunoreactive proteins.** In view of the apparent defects in insulin-stimulated [ $^3$ H]2-deoxyglucose uptake and activation of IRS-1-dependent PI3K and PKC- $\zeta$ , it was important to find that there were no significant differences in levels of immunoreactive IRS-1, PKC- $\zeta$ , and Glut4 glucose transporters in cultured adipocytes derived from lean and obese women (Fig. 4).

### Studies in myotubes

As reported previously (2), insulin-stimulated aPKC activity was diminished in myotubes derived from obese glucose-intolerant subjects (Fig. 5). Most interestingly, PIP<sub>3</sub> provoked insulin-like increases in aPKC activity in immunoprecipitates prepared from basal (*i.e.* not insulin stimulated) myotubes of lean controls (Fig. 5). In contrast, PIP<sub>3</sub> had a much lesser effect on aPKC activity in immunoprecipitates prepared from basal myotubes of obese glucose-intolerant subjects (Fig. 5). As might be expected from previous studies (4, 15, 19), aPKCs immunoprecipitated from lysates of insulin-stimulated myotubes of lean control subjects were not further activated by addition of PIP<sub>3</sub>, presumably reflecting maximal activation by previous insulin treatment. Similarly, aPKCs immunoprecipitated from lysates of insulin-stimulated myotubes of obese glucose-intolerant subjects were not further activated by addition of PIP<sub>3</sub>, indicating that the poor response to insulin was not simply due to a lack of PIP<sub>3</sub> generation.

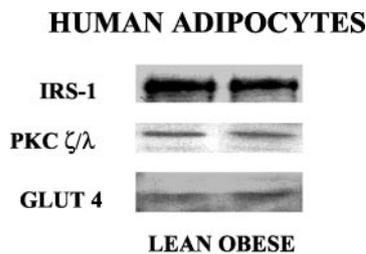


FIG. 4. Levels of IRS-1, PKC- $\zeta/\lambda$ , and GLUT4 glucose transporters in adipocytes derived from lean and obese women. Representative blots are shown here. Note that there were no significant differences in comparing multiple samples of obese subjects with multiple samples of lean subjects.

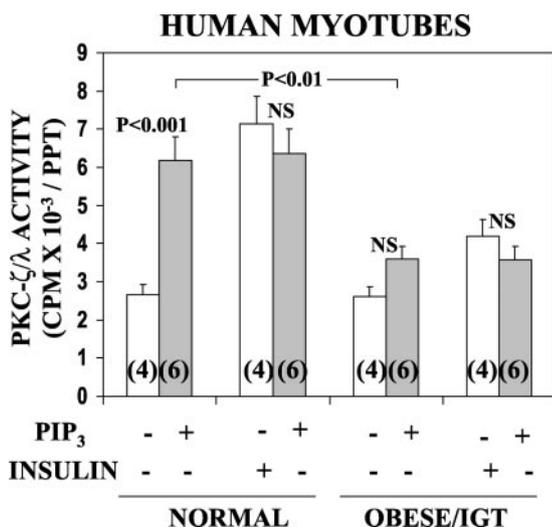


FIG. 5. Effects of insulin and PIP<sub>3</sub> on aPKC activity in cultured myotubes prepared from muscle cells taken from lean control and obese glucose-intolerant subjects. Myotubes were incubated for 10 min with or without 17 nM insulin, and aPKCs were immunoprecipitated and assayed in the presence or absence of 10  $\mu$ M PIP<sub>3</sub>, as indicated. Values are mean  $\pm$  SE of the number of determinations shown in parentheses.

### Discussion

It was surprising to find that, relative to cultured adipocytes of lean women, there were apparent defects in insulin-stimulated glucose transport and activation of IRS-1-dependent PI3K and aPKCs in cultured adipocytes derived from obese women. Because these adipocytes were differentiated from preadipocytes that had been passaged twice and subsequently cultured *in vitro*, these defects were clearly not dependent upon the continued presence of circulating factors, but, instead, were relatively stable and therefore seemed to be intrinsic. On the other hand, we cannot exclude the possibility that obesity-associated circulating factors may have induced intracellular changes that, for uncertain reasons, persisted through cell passaging and differentiation of preadipocytes to adipocytes.

In view of the marked defect in activation of IRS-1-dependent PI3K observed in adipocytes derived from obese women, it was surprising to find that PKB phosphorylation/activation was not significantly diminished in adipocytes derived from obese women. Interestingly, a similar, albeit less pronounced, dichotomy between insulin-stimulated IRS-1-dependent PI3K activity and PKB phosphorylation/activity has also been seen in muscles of obese, type 2 diabetic humans (14–16) and monkeys (19) and in adipocytes of type 2 diabetic rats (20). This dichotomy may reflect less of a dependence of PKB on IRS-1-dependent PI3K, or a greater degree of or full PKB activation may be effected by a relatively small amount of IRS-1-dependent PI3K activation. In keeping with the former explanation, *i.e.* less of a dependence on IRS-1, we have found that PKB activation is not compromised in either white adipocytes obtained from IRS-1 knockout mice or in immortalized brown adipocytes in which IRS-1 has been knocked out by homologous recombination, suggesting that factors other than IRS-1, *e.g.* IRS-2 and IRS-3, are sufficient for PKB activation in adipocytes (Miura, A., M. P. Sajan, G. Bandyopadhyay, M. L. Standaert, C. R. Kahn, and R. V. Farese, unpublished observations). On the other hand, as in the present studies in adipocytes of obese women in which IRS-1-dependent PI3K activation was markedly impaired, aPKC activation is partially compromised in white adipocytes of IRS-1 knockout mice and in immortalized brown adipocytes in which IRS-1 (or, for that matter, IRS-2) has been knocked out (Miura, A., M. P. Sajan, G. Bandyopadhyay, M. L. Standaert, C. R. Kahn, and R. V. Farese, unpublished observations). We believe that full activation of aPKCs in adipocytes is dependent on both IRS-1 and IRS-2, whereas PKB activation is fully activated by either IRS-1 or IRS-2, perhaps acting in conjunction with other PI3K activators.

The defect in PKC- $\zeta$  activation that was observed in adipocytes derived from obese women was probably largely secondary to the defect in IRS-1-dependent PI3K activation. However, the direct activation of PKC- $\zeta$  by PIP<sub>3</sub>, the lipid product of PI3K, was also defective, indicating a defect in one or more of the three known PIP<sub>3</sub>-dependent activating mechanisms (see Ref. 21), or in the intrinsic enzyme activity of PKC- $\zeta$ . Studies in other situations in which PIP<sub>3</sub> effects on aPKC activity are diminished (19, 22) in fact suggest that the initial PIP<sub>3</sub>-dependent activating step, *viz.*, phosphorylation of the aPKC activation loop site by 3-phosphoinositide-dependent protein kinase-1, is not compromised, suggesting that the defect is distal

to this step, *i.e.* in PIP<sub>3</sub>-dependent autophosphorylation, PIP<sub>3</sub>-dependent relief of autoinhibition by the regulatory pseudo-substrate sequence, or actual enzymatic activity of aPKCs. Further studies are needed to better elucidate the reason for unresponsiveness of aPKCs to PIP<sub>3</sub> and to see whether the defect in IRS-1-dependent PI3K activation may have been responsible for the defect in the activation of PKC- $\zeta$  by PIP<sub>3</sub>.

The presently observed defect in responsiveness of PKC- $\zeta$  to PIP<sub>3</sub> in adipocytes derived from obese women is noteworthy, because similar defects have been observed in skeletal muscles of obese and type 2 diabetic monkeys (19) and type 2 diabetic humans (15). In addition, a similar defect in aPKC responsiveness to PIP<sub>3</sub> has been observed in skeletal muscles of high-fat-fed rodents (22). Clearly, in fat-fed rodents, this defect in muscle aPKC responsiveness to PIP<sub>3</sub> is acquired, perhaps resulting from alterations in circulating or local levels of lipids/free fatty acids or adipocyte-derived endocrine/autocrine factors, such as resistin, cytokines, and/or adiponectin. Whether these factors were responsible for the defects in IRS-1-dependent PI3K and PKC- $\zeta$  activation remains to be determined.

Finally, it is noteworthy that, as presently seen in cultured adipocytes derived from obese women, persistent defects in insulin-stimulated glucose transport and activation of aPKCs, but not PKB, have been seen in passaged/cultured myotubes derived from muscles of obese glucose-intolerant subjects (2). Of further note, we presently found that PIP<sub>3</sub> provoked insulin-like increases in activity of aPKCs precipitated from myotubes derived from normal lean subjects but was much less effective in stimulating activity of aPKCs precipitated from myotubes derived from obese glucose-intolerant subjects. Thus, in both adipocytes of obese women and myotubes of obese/glucose-intolerant subjects, defects in glucose transport and aPKC activation are present and persist during cellular passaging, differentiation, and subsequent culture. These defects therefore appear to be independent of circulating extrinsic factors but may be due to autocrine or intracellular factors that are capable of altering insulin signaling to aPKCs. The similarity of these defects in different tissues raises the possibility that there is a common defect that alters aPKC activation and activity in adipocytes and muscle. Further studies are needed to elucidate the molecular mechanisms that are responsible for poor aPKC activation in adipocytes and myocytes of obese insulin-resistant subjects.

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