Hepatic insulin resistance in ob/ob mice involves increases in ceramide, aPKC activity, and selective impairment of Akt-dependent FoxO1 phosphorylation

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Abstract Pathogenesis of insulin resistance in leptin-deficient ob/ob mice is obscure. In another form of diet-dependent obesity, high-fat-fed mice, hepatic insulin resistance involves ceramide-induced activation of atypical protein kinase C (aPKC), which selectively impairs protein kinase B (Akt)-dependent forkhead box O1 protein (FoxO1) phosphorylation on scaffolding protein, 40 kDa WD(tryp-x-x-asp)-repeat propeller/FYVE protein (WD40/ProF), thereby increasing gluconeogenesis. Resultant hyperinsulinemia activates hepatic Akt and mammalian target of rapamycin (mTOR) pathways that lead to insulin resistance in high-fat-fed (HFF) mice (3–5), obese minimally-diabetic ob/ob mice, hepatic ceramide and aPKC activity and its association with WD40/ProF were increased. Hepatic Akt activity was also increased, but Akt associated with WD40/ProF was diminished and accounted for reduced FoxO1 phosphorylation and increased gluconeogenic enzyme expression. Most importantly, liver-selective inhibition of aPKC decreased aPKC and increased Akt association with WD40/ProF, thereby restoring FoxO1 phosphorylation and reducing gluconeogenic enzyme expression. Additionally, lipogenic enzyme expression diminished, and insulin signaling in muscle, glucose tolerance, obesity, hepatosteatosis, and hyperlipidemia improved. In conclusion, hepatic ceramide accumulates in response to CNS-dependent dietary excess irrespective of fat content; hepatic insulin resistance is prominent in ob/ob mice and involves aPKC-dependent displacement of Akt from WD40/ProF and subsequent impairment of FoxO1 phosphorylation and increased expression of hepatic gluconeogenic and lipogenic enzymes; and hepatic alterations diminish insulin signaling in muscle.—Sajan, M. P., R. A. Ivey, M. C. Lee, and R. V. Farese. Hepatic insulin resistance in ob/ob mice involves increases in ceramide, aPKC activity, and selective impairment of Akt-dependent FoxO1 phosphorylation. J. Lipid Res. 2015. 56: 70–80.

Insulin resistance with resultant hyperinsulinemia reflects an impairment in insulin-regulated glucose homeostasis, and is a key pathogenic element in obesity, metabolic syndrome features, and type 2 diabetes mellitus (T2DM). In this regard, primary impairments in insulin-stimulated glucose transport in muscle owing to muscle-specific knockout of the insulin receptor, the Glut4 glucose transporter, or atypical protein kinase C (aPKC), PKC-ζ can induce insulin resistance and development of obesity, metabolic syndrome, and T2DM (1, 2). However, dietary excesses appear to be particularly important contributors to the high prevalence of insulin-resistant disorders in Western/Westernized populations. Unfortunately, defects in glucose metabolism and underlying mechanisms that underlie insulin resistance in diet-induced obesity, and are only partly understood. Indeed, both the causes and required forms of dietary indiscretion that lead to insulin resistance are uncertain. In this regard, however, excessive activity of hepatic aPKC seems to be a commonly observed and critically important contributor to insulin resistance in high-fat-fed (HFF) mice (3–5), muscle-specific PKC-ζ knockout mice (6), obese overtly diabetic ob/ob mice (3, 7), and type 2 diabetic rats (3, 7) and humans (8).

Supplementary key words atypical protein kinase C • protein kinase C-ζ • protein kinase B • lipogenesis • gluconeogenesis • obesity • type 2 diabetes • ceramide • forkhead box O1 protein • mammalian target of rapamycin

This work was supported by funds from the Department of Veterans Affairs Merit Review Program and the National Institutes of Health Grants (7R01DK 065969-09) to R.V.F. The authors declare no financial conflicts of interest.

Received 17 July 2014 and in revised form 5 November 2014.

Published, JLR Papers in Press, November 13, 2014
DOI 10.1194/jlr.M052977

This article is available online at http://www.jlr.org

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Abbreviations: ACC, acetyl-coA carboxylase; ACPD, 2-acetyl-1,3-cyclopentanedione; Akt, protein kinase B; aPKC, atypical protein kinase Cζ; FoxO1, forkhead box O1 protein; G6Pase, glucose-6-phosphatase; GSK3β, glycogen synthase-3β; GTT, glucose tolerance test; HFF, high-fat-fed; NFκB, nuclear factor κB; mTOR, mammalian target of rapamycin; PEPCK, phosphoenolpyruvate carboxikinase; PI3K, phosphatidylinositol 3-kinase; SREBP-1c, sterol receptor binding protein-1c; T2DM, type 2 diabetes mellitus; WD40/ProF, 40 kDa WD(tryp-x-x-asp)-repeat propeller/FYVE protein.

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In many studies of diet-related insulin resistance, mice are fed an artificial diet containing excessive amounts of fat, most commonly supplying 60% of calories from fat, instead of the usual 10% in standard mouse chow. These HFF mice may have impairment in insulin-stimulated hepatic protein kinase B (Akt) activity (9–11), and such an impairment could reasonably account for decreases in phosphorylation of the forkhead box O1 protein (FoxO1), which mediates insulin/Akt effects on gluconeogenic enzymes, i.e., decreases in expression of phosphoenolpyruvate carboxykinase (PEPCK) and glucose-6-phosphatase (G6Pase) (12, 13). Increases in hepatic glucose output contribute strongly to insulin resistance in these mice. However, in similar studies of these HFF mice, increases in resting/basal hepatic Akt activity have been noted by some investigators (14, 15) (our experience is similar). Moreover, in HFF mice consuming a moderate-high fat “Western” diet that supplies 40% of calories from milk fat, there are clear increases in both resting/basal and insulin-stimulated activity of hepatic Akt2, presumably reflecting hyperinsulinemia and increased activation of upstream activators of Akt2, namely, insulin receptor substrate (IRS)-1- and/or IRS-2-dependent phosphatidylinositol 3-kinase (PI3K) (5). Nevertheless, despite the overall elevation of hepatic Akt2 activity in these HFF mice, FoxO1 phosphorylation is diminished and expression of PEPCK and G6Pase is elevated (5). This decrease in hepatic Akt-dependent FoxO1 phosphorylation in these HFF mice is relatively specific, as phosphorylation of other hepatic Akt substrates, glycogen synthase-3B (GSK3B), which increases glycogenesis, and mammalian target of rapamycin (mTOR)C1, which increases lipogenesis (16), mirror the alterations in total cellular Akt2 activity, i.e., they are increased (5).

The selective defect in Akt-dependent phosphorylation of FoxO1 in the livers of HFF mice appears to be provoked by increases in hepatic aPKC activity that are caused by: a) increased hepatic levels of ceramide (and possibly phosphatidic acid), which, like phosphatidylinositol-3,5-(Pi)3, (PIP3), directly activates aPKC; and b) hyperinsulinemia (5). In support of this idea: a) FoxO1 phosphorylation is selectively facilitated by colocalization of insulin-activated Akt2 and FoxO1 on scaffolding protein, 40 kDa WD(tryp-x-asp)-repeat propeller/FIVê protein (WD40/ProF) (5, 17–19); b) activated aPKC is similarly colocalized on the WD40/ProF platform (5, 17–19); c) WD40/ProF-associated aPKC levels and activity are excessive in the livers of HFF mice (5); d) aPKC can bind (20), phosphorylate (21), and directly inhibit (22) Akt; e) WD40/ProF-associated Akt2 activity is diminished in the livers of HFF mice (5); and f) most importantly, selective inhibition of hepatic aPKC in HFF mice diminishes aPKC association with WD40/ProF, and this is accompanied by increases in WD40/ProF-associated Akt2 activity, increases in FoxO1 phosphorylation, and decreases in PEPCK and G6Pase expression (5). Collectively, these findings suggest that excessive activation of hepatic aPKC in HFF mice diminishes Akt2 activity (or its actual presence, as shown here) on the WD40/ProF platform and this alteration diminishes FoxO1 phosphorylation and thereby increases PEPCK and G6Pase expression. Moreover, Akt-dependent phosphorylation of mTORC1 is excessive in response to hyperinsulinemia in these HFF mice (5, 13, 14), and, in conjunction with hyperactive hepatic aPKC (23), provokes the activation of sterol receptor binding protein-1c (SREBP-1c) and nuclear factor κB (NFκB), and subsequent increases in expression of lipogenic enzymes and proinflammatory cytokines (5). These abnormalities presumably increase hepatic output of glucose, lipids, and proinflammatory cytokines; and these and/or other liver-dependent abnormalities appear to be responsible for diminished insulin signaling in muscle in these HFF mice (5). It was therefore postulated that, in HFF mice, inordinate increases in hepatic aPKC activity are provoked initially by ceramide and secondarily by hyperinsulinemia following increases in hepatic gluconeogenesis (5).

In view of the aforesaid findings in HFF mice, we questioned whether a similar situation exists in diet-induced obesity models that do not involve high-fat feeding. For this purpose, we used ob/ob mice, as these mice genetically lack the CNS appetite-suppressant, leptin, and develop obesity, metabolic syndrome, and T2DM while eating excessive quantities of standard low-fat mouse chow. On the other hand, we previously evaluated 8–12-week-old overtly diabetic ob/ob mice and found that they have markedly impaired activation of hepatic Akt, but elevated activation of hepatic aPKC (3, 7). However, here, we took advantage of the fact that ob/ob mice generated on a C57Bl/6 background are at first markedly diabetic, but, after several months, their diabetes is largely ameliorated by a surge in pancreatic insulin production. Nevertheless, these minimally diabetic ob/ob mice retain their insulin resistance, obesity, and other metabolic syndrome features. Moreover, as we presently found, these obese-phase ob/ob mice have hepatic insulin signaling abnormalities that are much less advanced than those present in overtly diabetic ob/ob mice.

Presently, we studied these obese-phase ob/ob mice over 10 weeks, during which they consumed low-fat chow, with or without an agent that selectively targets and inhibits hepatic aPKC (5, 24). We found that these ob/ob mice are different from younger overtly diabetic ob/ob mice in that hepatic Akt activity/activation is clearly excessive, rather than diminished. On the other hand, the obese-phase ob/ob mice are similar to HFF mice fed a diet supplying 40% of calories from milk fat, not only in that hepatic Akt activity/activation is elevated, but also in that insulin resistance involves similar increases in hepatic ceramide content, aPKC-dependent displacement of Akt from the WD40/ProF platform, inhibition of Akt-mediated FoxO1 phosphorylation (that normally occurs on the WD40/ProF platform), subsequent increases in expression of gluconeogenic, lipogenic, and proinflammatory factors, and secondary impairment in insulin signaling to aPKC and Akt in muscle. Moreover, inhibition of hepatic aPKC largely prevented or reversed hepatic and muscle abnormalities, and substantially improved glucose intolerance and lipid abnormalities in these obese-phase ob/ob mice.
aPKC inhibitor

Inhibitor of aPKCs, PKCα, and PKCζ, 2-acetyl-1,3-cyclopentanetanedione (ACPĐ), was purchased from Sigma (St. Louis, MO). Its specificity was reported previously (5, 24). In addition, we presently found that ACPĐ did not inhibit kinases, Akt2, FGFR1/2/3/4, mTOR, GSK3β, IRAK1/4, JAK1/2, MEK1, ERK1/2, JNK1/2, PAK, Src, ROCK2, ROS1, or PI3Kα/α, as tested by Life Technologies (Madison WI).

Mouse studies

Male (C57Bl/6) ob/ob and lean ob+ mice (Jackson Laboratory, Bar Harbor, ME) were studied over 10 weeks between the ages of 3 and 5 months, during which time they were injected subcutaneously daily with ACPĐ (10 mg/kg body weight) in saline or saline/vehicle. At 9 weeks, glucose tolerance was measured after an overnight fast by intraperitoneal injection of 2 mg glucose per kilogram body weight, as described (2, 5). At week 10, mice were treated acutely for 15 min with insulin (1 U/kg body weight) prior to euthanization. In an ancillary study, C57Bl/6 mice were placed on a diet providing 60% of calories from fat (Teklad) and treated daily as above in ob/ob mice, with saline or ACPĐ.

All experimental procedures involving animals were approved by the Institutional Animal Care and Use Committees of the University of South Florida College of Medicine and the James A. Haley Veterans Administration Medical Center Research and Development Committee, Tampa, FL.

Study of lipid synthesis in human hepatocytes

As described (8, 24), hepatocytes of lean, obese, and type 2 diabetic humans were incubated overnight and then incubated for 4 h in the presence of 2 μCi uniformly-labeled 14C-acetate (Perkin Elmer NEN) and 10 μM sodium acetate carrier, and, where indicated, 2 μM ACPĐ; following which, tissue lipids were extracted by the method of Bligh and Dyer and counted for radioactivity.

Tissue preparations

Liver and muscle samples were homogenized as described (3–8, 24). Nuclei were isolated as described (4–7).

aPKC activity and Akt activity/activation

aPKC activity/activity was assayed by immunoblotting for phosphorylation of the auto(trans)phosphorylation site, thr-555/560 in PKCζ, which is required for, and reflective of, activation (25). Akt activity/activation was assayed by immunoblotting for phosphorylation of ser-473-Akt.

Western analyses

Western analyses were conducted as described (3–8, 24) using: rabbit polyclonal anti-phospho-serine-473-Akt, anti-GAPDH, anti-WD40/ProF, anti-aPKC antiserum, anti-FAS, anti-GαPase, anti-PEPCK, anti-p65/RelA/NFκB antiserum (Santa Cruz Biotechnology, Santa Cruz, CA); rabbit polyclonal anti-phospho-threonine-560/555-PKCζ/α antiserum (Invitrogen, Carlsbad, CA); rabbit polyclonal anti-p-serine-256-FoxO1 and anti-FoxO1 antiserum (Abnova, Walnut, CA); mouse monoclonal anti-PKCα/α antibodies (Transduction Antibodies, Bedford, MA); rabbit polyclonal anti-phospho-serine-9-GSK3β, anti-acetyl-coA carboxylase (ACC) and anti-phospho-serine-2248-mTOR antiserum, and mouse monoclonal anti-Akt antibodies (Cell Signaling Technologies, Danvers, MA); mouse monoclonal anti-SREBP-1 antibodies (Lab Vision Corp., Freemont, CA); horseradish-peroxidase-conjugated goat anti-mouse and anti-rabbit secondary antibodies (Bio-Rad, Hercules, CA); and horseradish-peroxidase-conjugated AfiniPure donkey anti-mouse and anti-rabbit secondary antibodies (Jackson Immuno-Research Labs, West Grove, PA). Samples from experimental groups were compared on the same blots and routinely checked with loading controls.

Measurements of serum triglycerides, cholesterol, free fatty acids, insulin, and glucose

Serum triglycerides, insulin, and glucose levels were measured as described (2, 5, 6).

Liver triglycerides

As described (2), triglycerides were extracted from liver lysates by the method of Bligh and Dyer and quantified using a kit from Sigma-Aldrich (catalog number TR0100).

mRNA measurements

Methods for measurement of mRNA were described previously (4–7).

Ceramide species quantification

Ceramide species were measured by liquid chromatography tandem mass spectrometry analysis of liver extracts of lean and ob/ob mice by Lipidomics Shared Resource, Medical University of South Carolina (Charleston, SC).

Statistical evaluations

Data are expressed as mean ± SEM, and P values were determined by one-way ANOVA and least-significant multiple comparison methods.

RESULTS

Phosphorylation/activity of aPKC and Akt in liver and muscle of lean and ob/ob mice

In lean control ob+ mice, insulin increased phosphorylation of both aPKC and Akt in liver and muscle (Fig. 1a–d). In muscles of ob/ob mice, however, resting/basal and insulin-stimulated increases in phosphorylation of aPKC were significantly diminished, and insulin-stimulated Akt phosphorylation trended downward relative to findings in lean ob+ mice (Fig. 1b,d).

In contrast to muscle, phosphorylation of hepatic aPKC in ob/ob mice was increased in resting/basal conditions to a level comparable to, if not greater than, that seen with insulin treatment in lean ob+ mice, and acute exogenous insulin treatment had no further effect on aPKC phosphorylation (Fig. 1c), suggesting that hepatic aPKC was maximally activated in obese-phase ob/ob mice by endogenous hyperinsulinemia (see below) and/or other factors. Partly similar to hepatic aPKC, resting/basal hepatic Akt phosphorylation was elevated in ob/ob mice and, with acute exogenous insulin treatment, Akt phosphorylation was elevated to levels not significantly different from those seen in lean ob+ mice (Fig. 1a).

Effects of ACPĐ on phosphorylation (activation) of aPKC and Akt in liver and muscle of lean and ob/ob mice

Treatment of ob/ob mice with ACPĐ reduced the elevated resting/basal levels of hepatic aPKC phosphorylation to nearly normal, and substantially diminished stimulatory effects of exogenous insulin treatment on hepatic aPKC.
aPKC inhibits hepatic FoxO1 phosphorylation in ob/ob mice

Phosphorylation/activities of aPKC and Akt in resting/basal and insulin-stimulated conditions and during treatment with aPKC inhibitor in liver (A, C) and muscle (B, D) lysates of lean ob+ and obese-phase ob/ob mice. Over 10 weeks, lean ob+ mice and ob/ob mice were injected subcutaneously daily with 0.2 ml physiologic saline or saline containing aPKC inhibitor, ACPD (10 mg/kg body weight). Before euthanization, ad libitum-fed mice were treated for 15 min with or without insulin (1 U/kg body weight, intraperitoneally). Values are mean ± SEM of (N) determinations. *P < 0.05; **P < 0.01; ***P < 0.001.

Phosphorylation of FoxO1, mTOR, and GSK3β in liver of lean and ob/ob mice and effects of ACPD treatment

In lean ob+ mice, in conjunction with increases in Akt phosphorylation, insulin increased hepatic FoxO1 phosphorylation (Fig. 2a). In ob/ob mice, however, despite hyperinsulinemia and elevated levels of hepatic Akt phosphorylation in resting/basal and insulin-stimulated conditions, FoxO1 phosphorylation remained at a lower or modestly diminished resting/basal level and, moreover, responded poorly if at all to acute insulin treatment (Fig. 2a). However, with ACPD treatment of ob/ob mice, both resting/basal and insulin-stimulated FoxO1 phosphorylation increased to levels comparable to those seen in insulin-stimulated lean ob+ mice (Fig. 2a).

In marked contrast to FoxO1, phosphorylation of both mTOR and GSK3β was increased, not only by insulin in lean ob+ mice, but also in resting/basal conditions (presumably reflecting hyperinsulinemia and increased Akt activation), and following exogenous insulin treatment, in ob/ob mice (Fig. 2b, c). Moreover, ACPD treatment did not substantially alter the elevations in mTORC and GSK3β phosphorylation seen in ob/ob mice (Fig. 2b, c).

Association of aPKC and Akt on WD40/ProF in lean ob+ and ob/ob mice

Insulin acutely increased recovery of aPKC and Akt in WD40/ProF immunoprecipitates (Fig. 3a, b). In ob/ob phosphorylation (Fig. 1c). In contrast to aPKC, hepatic Akt phosphorylation in ob/ob mice was not altered significantly by ACPD treatment (Fig. 1a).

Very interestingly, impairments in insulin-stimulated phosphorylation of both aPKC and Akt in muscles of ob/ob mice were significantly improved or trended upward with ACPD treatment (Fig. 1b, d).
mice, however, the resting/basal level of aPKC associated with WD40/ProF was increased maximally, as it did not increase further with insulin treatment (Fig. 3a); in contrast, Akt associated with WD40/ProF was diminished basally and increased poorly following insulin treatment (Fig. 3b). Most interestingly, ACPD treatment diminished aPKC association with WD40/ProF (Fig. 3a), and this was accompanied by increased association of Akt with WD40/ProF (Fig. 3b).

In the above-described studies, basal hepatic Akt activity was elevated in ob/ob mice and could have contributed to increases in Akt association with WD40/ProF and subsequent FoxO1 phosphorylation following ACPD treatment. However, in mice placed on a 60% high-fat diet, basal Akt activity was not significantly increased above that of low-fat-fed mice (1.15 ± 0.15 vs. 0.88 ± 0.09; mean ± SEM), and we observed similar increases in Akt association with WD40/ProF and FoxO1 phosphorylation, despite the fact that Akt phosphorylation, if anything, trended downward with ACPD treatment (Fig. 4). Thus, the ACPD-induced decrease in aPKC activity and its diminished association with WD40/ProF itself appeared to be sufficient to alter Akt association and FoxO1 phosphorylation.

Expression of gluconeogenic, lipogenic, and proinflammatory factors in livers of ob/ob mice

In ob/ob mice, hepatic expression (mRNA) of gluconeogenic enzymes, PEPCK and G6Pase, lipogenic enzymes, SREBP-1c, FAS and ACC, and proinflammatory TNF-α were elevated, but virtually restored to normal with ACPD treatment (Fig. 5). Similarly, in ob/ob mice, nuclear protein levels of the active fragment of SREBP-1c and nuclear protein levels of the active p65/RelA subunit of NFκB were increased (Fig. 6a), and lysate protein levels of ACC, FAS, PEPCK, and G6Pase were increased, and ACPD treatment largely reversed these increases (Fig. 6b).

To determine whether ACPD-induced decreases in expression of lipogenic enzymes are accompanied by actual decreases in lipid synthesis, we incubated hepatocytes of obese and type 2 diabetic humans in which there were...
2- to 4-fold elevations in SREBP-1c, FAS, and ACC (8, 24), and we found that $^{14}$C-acetate incorporation into lipids during a 4 h pulse was markedly increased in hepatocytes of obese and type 2 diabetic humans, and these increases were largely reversed by ACPD treatment [45 ± 6, lean-ACPD; 558 ± 150, obese-ACPD; 388 ± 171, T2DM-ACPD; and with ACPD treatment, 43 ± 9, lean+ACPD; 62 ± 9, obese+ACPD; 55 ± 4, T2DM+ACPD (all values are mean ± SE cpm/10^6 cells; N = 6)].

**Glucose tolerance and lipid parameters in ob/ob mice**

Fasting blood glucose levels were only modestly elevated in ob/ob mice relative to lean ob⁻ control mice (note that blood glucose levels are much higher in diabetic-phase

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**Fig. 3.** Association of aPKC (A) and Akt (B) with hepatic WD40/ProF in resting/basal and insulin-stimulated conditions, and during treatment with aPKC inhibitor, ACPD, in lean ob⁻ and obese ob/ob mice. Mice were treated with ACPD and insulin as in Fig. 1. WD40/ProF was immunoprecipitated from liver lysates. Values are mean ± SEM of (N) determinations. *P < 0.05; **P < 0.01; ***P < 0.001. Representative blots are shown; note unchanged level of WD40/ProF in immunoprecipitates.

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**Fig. 4.** Effects of treatment with aPKC inhibitor, ACPD, on activities of aPKC and Akt, association of aPKC and Akt with WD40/ProF, and FoxO1 phosphorylation in HFF mice. Mice were placed on a diet providing 60% of calories from fat and treated without (clear bars) or with (shaded bars) ACPD (10 mg/kg body weight) for 10 weeks. Liver lysates were analyzed for indicated parameters. Values are mean ± SEM of eight determinations. *P < 0.05. Representative blots are shown; note unchanged level of FoxO1 in immunoprecipitates.
As expected, food intake was increased in ob/ob mice, and ACPD treatment had little or no effect on food intake (Fig. 8b). On the other hand, increases in body weight during the 10 week study period trended downward in ob/ob mice with ACPD treatment (Fig. 8a). Of further note, in ob/ob mice, combined weights of epididymal plus retroperitoneal fat depots (Fig. 8c), and serum (Fig. 8d) and hepatic triglyceride levels (Fig. 8e) diminished following ACPD treatment. The decrease in liver triglycerides

ob/ob mice); on the other hand, glucose tolerance, as measured in the peritoneal glucose tolerance test (GTT), was more substantially compromised (Fig. 7). Most importantly, treatment of ob/ob mice with ACPD led to substantial improvements in both fasting blood glucose levels and glucose tolerance, as measured in the GTT (Fig. 7). On the other hand, the elevated fasting and post-glucose insulin levels seen in ob/ob mice during the GTT, showed no significant change with ACPD treatment (Fig. 7, inset).

Fig. 5. Effects of treatment with aPKC inhibitor, ACPD, on mRNA levels of hepatic SREBP-1c, FAS, ACC, TNF-α, PEPCK, and G6Pase in obese ob/ob mice. Mice were treated with ACPD and insulin as in Fig. 1. Acute 15 min insulin treatment did not alter mRNA levels. Values are mean ± SEM of (N) determinations. *P < 0.05; **P < 0.01; ***P < 0.001.

Fig. 6. Effects of treatment with aPKC inhibitor, ACPD, on hepatic nuclear levels of the active 60–70 kDa proteolytic fragment of SREBP-1c and the active p65/RelA subunit of NFκB (a), and on hepatic lysate protein levels of FAS, ACC, PEPCK, and G6Pase in obese ob/ob mice (b). Mice were treated with ACPD and insulin as in Fig. 1. Acute 15 min insulin treatment did not alter protein levels. Values are mean ± SEM of (N) determinations. *P < 0.05; **P < 0.01; ***P < 0.001.
levels of various ceramide species and sphingomyelin, a potential precursor of ceramide, were increased in livers of ob/ob mice relative to lean ob' control mice (Fig. 9).

**DISCUSSION**

It was important to find in ob/ob mice that, relative to ob' lean control mice, hepatic ceramide levels and aPKC activity were increased, as ceramide appeared to partially account for increases in aPKC activity in HFF mice (5). It was equally important to find that hepatic Akt activity was elevated in the resting/basal state and increased to levels comparable to those seen in lean ob' mice following insulin treatment of ob/ob mice. The latter findings suggested that resting/basal activities of Akt activity in ob/ob mice were increased by hyperinsulinemia, and Akt responsive-ness to insulin was largely intact. In keeping with this idea, the phosphorylation of two Akt substrates, mTORC and GSK3β, was elevated to levels comparable to the elevation in Akt activity. Needless to say, the hyperinsulinemia most likely contributed, along with ceramide, to excessive activation of aPKC in ob/ob mice.

The finding of elevated resting/basal and normal insulin-stimulated hepatic Akt activity in presently used 5-month-old ob/ob mice might seem at odds with previous findings showing that 2–3-month-old overtly diabetic ob/ob mice have low resting/basal and markedly diminished insulin-stimulated hepatic Akt activity (3, 7). However, as discussed, an age-dependent amelioration of the diabetic state occurs at about the age of the ob/ob mice presently used, owing to an age-dependent compensatory increase in pancreatic

As ceramide potently activates aPKC, and, as ceramide is increased in HFF mice (5), it was important to find that

![Graph showing blood glucose levels](image)

**Fig. 7.** Effects of treatment with aPKC inhibitor, ACPD, on glucose tolerance in obese ob/ob mice. Mice were treated with ACPD and insulin as in Fig. 1, except, at 9 weeks, after overnight fast, glucose tolerance was tested. Values are mean ± SEM of nine to twelve determinations. *P < 0.05; **P < 0.01; ***P < 0.001, ACPD-untreated ob/ob versus ob' mice. †††P < 0.001. Inset shows serum insulin levels.

 induced by ACPD may reflect diminished synthesis owing to diminished SREBP-1c action on lipogenic enzymes, coupled with increased fatty acid oxidation owing to diminished levels of ACC.

**Ceramide and sphingomyelin levels in livers of obese ob/ob and lean ob' control mice**

![Graphs showing body weight, food intake, and serum triglycerides](image)

**Fig. 8.** Effects of aPKC inhibitor, ACPD, on alterations in body weight (a), food intake (b), combined weight of epididymal plus retroperitoneal fat (c), serum triglycerides (d), and liver triglycerides (e) in ob/ob mice. Mice were treated with ACPD and insulin as in Fig. 1. Acute 15 min insulin treatment did not alter these parameters. Values are mean ± SEM of (N) determinations. *P < 0.05; **P < 0.01; ***P < 0.001.
It may be noted that we previously reported that Akt2 activity associated with WD40/ProF was diminished in HFF mice. Presently, by scaling up the amount of liver lysate used for immunoprecipitation, and by using mouse monoclonal anti-Akt primary antibody and donkey immunodepleted secondary antibody, we were able to detect actual decreases in Akt levels associated with Wd40/ProF in ob/ob mice, and a restoration of these levels with inhibition of hepatic aPKC. Whether the close proximity of aPKC and Akt on the WD40/ProF platform facilitated Akt phosphorylation and subsequent release from the platform, or whether the aPKC alters the phosphorylation state of the WD40/ProF platform and thereby limits Akt access is uncertain. In this regard, we have found that hepatic aPKC activity correlates inversely with FoxO1 phosphorylation in many instances (4–6, 24, 26), including the fasting state (4), and it appears that aPKC tonically limits FoxO1 phosphorylation and thereby promotes hepatic gluconeogenesis. In this scenario, aPKC may be useful in preventing hypoglycemia while fasting, but, on the other hand, would promote insulin resistance when excessively activated.

It is noteworthy that the improvement in the ability of insulin to increase FoxO1 phosphorylation with inhibition of hepatic aPKC occurred with little or no alteration in overall hepatic Akt activation. This lends further weight to the argument that the increase in Akt association with WD40/ProF during aPKC inhibitor treatment was caused by the decrease in aPKC levels associated with WD40/ProF owing to diminished activity of hepatic aPKC.

As treatment with aPKC inhibitor, ACPD, improved glucose tolerance in ob/ob mice with little or no alteration in serum insulin levels during the GTT, it may be surmised that: (a) improved glucose tolerance in ACPD-treated ob/ob mice was not due to increased insulin secretion; (b) insulin secretion was not compromised by prolonged ACPD treatment; and (c) residual insulin resistance continued following ACPD treatment. Nevertheless, from the improvements in glucose tolerance, hepatic FoxO1 phosphorylation, and insulin signaling in muscle, it seems clear that responsiveness to insulin was improved in both liver and muscle by ACPD treatment. Also note that: (a) we did not use higher doses of ACPD to see if they would further diminish hepatic aPKC activity and serum insulin levels; and (b) factors other than aPKC, such as increases in activities of other PKCs that impair insulin receptor function or otherwise compromise insulin effectiveness, or other factors, such as impaired thermogenesis, etc., may have contributed to residual insulin resistance in ACPD-treated ob/ob mice. Further studies of ACPD dosage and involvement of factors other than aPKC are needed to resolve these issues.

In addition to improvements in FoxO1 phosphorylation and gluconeogenic enzyme expression/abundance, the excessive activity of SREBP-1c and expression/expression of lipogenic enzymes, SREBP-1c, FAS, and ACC, and the excessive activity of NFκB and expression of proinflammatory TNF-α and/or other cytokines, improved with ACPD treatment. As overall hepatic Akt activity and activation
were not altered by ACPD, these alterations in lipogenic and proinflammatory gene function most likely reflect diminished activity of hepatic aPKC, which is required for insulin-induced activation of SREBP-1c and NFkB, and subsequent increases in expression of lipogenic and proinflammatory factors (4–8, 27–29). In this regard, note that the phosphorylation of mTOR, which mediates the stimulatory effects of Akt on lipogenesis during insulin action (14), was elevated basally and during insulin treatment in parallel with increases in hepatic Akt activity. Also note that the dichotomy between the impairment in Akt-dependent FoxO1 phosphorylation and the increases in Akt-dependent mTOR phosphorylation seen here in ob/ob mice and previously in HFF mice (5) helps to explain the paradox in insulin-resistant states that hepatic gluconeogenesis and lipogenesis are simultaneously elevated, despite the fact that simple insulin resistance would predict increases in gluconeogenesis and decreases in lipogenesis.

It seems likely that improvements in insulin signaling in muscle following treatment with inhibition of hepatic aPKC contributed importantly to the improvement in glucose tolerance in ob/ob mice, as glucose disposal is an important determinant of glucose levels in the GTT. Moreover, the fact that muscle aPKC activity increased in response to inhibition of hepatic aPKC suggests that improvements in liver factors were at least partly responsible for improvements in muscle aPKC activation by insulin. [Note that we previously reported (supplemental data to Ref. 5) that ACPD does not inhibit basal or insulin-stimulated aPKC in muscles of normal mice, suggesting that ACPD, as presently used, has little or no direct effect on muscle.] This suggests that hepatic release of lipids, proinflammatory cytokines, and/or other factors may diminish insulin signaling in muscle in ob/ob mice.

To summarize, the present findings show that diminished FoxO1 phosphorylation, owing to an excessive activation of hepatic aPKC and aPKC-dependent displacement of Akt from the WD40/ProF platform, is an important mechanism for increasing hepatic expression and abundance of gluconeogenic enzymes, and thereby provoking systemic insulin resistance in ob/ob mice consuming excessive amounts of a diet low in fat content. Thus, regardless of dietary fat content, and regardless of whether the initial cause of over-nutrition is deficient operation of appetite suppression in the CNS, simple caloric excess can lead to inordinate increases in hepatic lipids, such as ceramide, that directly activate hepatic aPKC and thereby provoke hepatic insulin resistance 45.

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