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Correction of metabolic abnormalities in a rodent model of obesity, metabolic syndrome, and type 2 diabetes mellitus by inhibitors of hepatic protein kinase C–ι

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ABSTRACT

Excessive activity of hepatic atypical protein kinase (aPKC) is proposed to play a critical role in mediating lipid and carbohydrate abnormalities in obesity, the metabolic syndrome, and type 2 diabetes mellitus. In previous studies of rodent models of obesity and type 2 diabetes mellitus, adenoviral-mediated expression of kinase-inactive aPKC rapidly reversed or markedly improved most if not all metabolic abnormalities. Here, we examined effects of 2 newly developed small-molecule PKC-ι/λ inhibitors. We used the mouse model of heterozygous muscle-specific knockout of PKC-λ, in which partial deficiency of muscle PKC-λ impairs glucose transport in muscle and thereby causes glucose intolerance and hyperinsulinemia, which, via hepatic aPKC activation, leads to abdominal obesity, hepatosteatosis, hypertriglyceridemia, and hypercholesterolemia. One inhibitor, 1H-imidazole-4-carboxamide, 5-amino-1-[2,3-dihydroxy-4-[(phosphonooxy)methyl]cyclopentyl-[1R-(1a,2b,3b,4a)], binds to the substrate-binding site of PKC-λ/ι, but not other PKCs. The other inhibitor, aurothiomalate, binds to cysteine residues in the PB1-binding domains of aPKC-λ/ι/ζ and inhibits scaffolding. Treatment with either inhibitor for 7 days inhibited aPKC, but not Akt, in liver and concomitantly improved insulin signaling to Akt and aPKC in muscle and adipocytes. Moreover, both inhibitors diminished excessive expression of hepatic, aPKC-dependent lipogenic, proinflammatory, and gluconeogenic factors; and this was accompanied by reversal or marked improvements in hyperglycemia, hyperinsulinemia, abdominal obesity, hepatosteatosis, hypertriglyceridemia, and hypercholesterolemia. Our findings highlight the pathogenetic importance of insulin signaling to hepatic PKC-ι in

MP Sajan oversaw conduct of all laboratory studies, supervised laboratory personnel, performed assays, and collected and analyzed data. S Nimal conducted laboratory assays. S Mastorides provided resources for measuring clinical parameters. M Acevedo-Duncan provided ICAPP. AL Fields provided ATM. CR Kahn and M Leitges provided original MCK-Cre transgenic and PKC-λ floxed mice, respectively, used for generation of the mouse colony. RV Farese provided overall planning and direction of the studies, analyzed data, and wrote the paper.

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obesity, the metabolic syndrome, and type 2 diabetes mellitus and suggest that 1H-imidazole-4-carboxamide, 5-amino-1-[2,3-dihydroxy-4-[(phosphonooxy)methyl]cyclopentyl-[1R-(1a,2b,3b,4a)] and aurothiomalate or similar agents that selectively inhibit hepatic aPKC may be useful treatments.

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1. Introduction

Obesity, metabolic syndrome, and type 2 diabetes mellitus are preeminent health problems. Abnormalities in these interrelated insulin-resistant disorders, including obesity, dyslipidemias, and glucose intolerance, are usually treated piecemeal and with limited success. Clearly, new approaches are needed to contain this pandemic. Identifying a unifying treatable pathogenetic factor would simplify this task.

Insulin controls metabolic processes by activating Akt and atypical protein kinase C (aPKC), which function distal to insulin receptor substrate (IRS)-1– and IRS-2–dependent phosphatidylinositol 3-kinase (PI3K). In rodent models of obesity and type 2 diabetes mellitus, hepatic aPKC activation by insulin is conserved, even when hepatic Akt activation is markedly diminished, as in advanced diabetes [1-3]. Branching of insulin signaling to Akt and aPKC pathways in diabetic liver [1-3] appears to reflect downregulated IRS-1/PI3K, which is a major factor in hepatic Akt activation [4-7], as opposed to conserved or heightened (by hyperinsulinemia) activation of IRS-2/PI3K, which alone mediates insulin activation of hepatic aPKC [4,6,7]. This branching of insulin signaling in liver contrasts with the situation in muscle, where IRS-1/PI3K controls both Akt and aPKC [5,6], which together control glucose transport and which together are downregulated in various forms of obesity and diabetes [8].

Conserved activation of hepatic aPKC in obesity, the metabolic syndrome, and type 2 diabetes mellitus is problematic, as hyperinsulinemia therein provokes excessive activation of hepatic aPKC and aPKC-dependent processes, including (*a*) expression of sterol receptor element binding protein–1c (SREBP-1c), which transactivates an array of lipogenic genes, for example, fatty acid synthase (FAS) and acetyl-CoA carboxylase (ACC) [2,3,9,10], and (*b*) activation of inhibitor of κB kinase-β which phosphorylates and inactivates inhibitor of nuclear factor κB-β, the inhibitor of nuclear factor κ-B (NFκB), thus releasing NFκB for nuclear uptake and transactivation of proinflammatory genes, for example, tissue necrosis factor–α (TNF-α) and interleukin-1β (IL-1β) [2,3,10].

In support of the idea that activation of hepatic aPKC, SREBP-1c, and NFκB in hyperinsulinemic states of obesity and type 2 diabetes mellitus contributes importantly to the development of hepatosteatosis, hypertriglyceridemia, hypercholesterolemia, impaired insulin signaling in muscle, and systemic insulin resistance, tissue-selective inhibition of hepatic aPKC by adenoviral-mediated expression of kinase-inactive aPKC or shRNA to knockdown hepatic IRS-2 diminishes aPKC activity and activation of SREBP-1c–dependent lipogenic and NFκB-dependent proinflammatory pathways [2,3]. In addition, adenoviral-mediated inhibition of hepatic aPKC diminishes fasting-dependent expression of gluconeogenic enzymes, phosphoenolpyruvate carboxykinase (PEPCK), and glucose-6-phosphatase (G6Pase) [2]. As a result of these alterations in liver enzymes, both adenoviral treatments rapidly, that is, over the course of 5 days, reverse or markedly improve the above-mentioned clinical abnormalities in several rodent models of obesity and type 2 diabetes mellitus [2,3].

Here, we examined effects of 2 newly developed small-molecule inhibitors of the aPKC, PKC-λ/ι, on insulin signaling and activation of lipogenic, proinflammatory, and gluconeogenic pathways in livers of obese mice with type 2 diabetes mellitus. In this model [10], in response to gene knockout-induced partial (heterozygous) deficiency of aPKC in muscle, there is a specific impairment of glucose transport in muscle, which is followed by development of glucose intolerance, insulin resistance, and hyperinsulinemia, which in turn leads to excessive activation of hepatic aPKC and aPKC-dependent lipogenic, proinflammatory, and gluconeogenic pathways, which is ultimately followed by development of clinical abnormalities, that is, abdominal obesity, hepatosteatosis, hypertriglyceridemia, and hypercholesterolemia. As described below, both chemical inhibitors rapidly and largely reversed all hepatic and clinical abnormalities.

2. Methods

2.1. PKC-λ/ι inhibitors

Aurothio**m**alate (ATM) was purchased from commercial sources as the pharmaceutical product known as *Monochrysine*, produced by Taylor Pharmaceuticals (Decatur, IL). 1H-**i**midazole-4-**c**arboxamide, 5-**a**mino-1-[2,3-dihydroxy-4-[(**p**hosphono-oxy)methyl]cyclo**p**entyl-[1R-(1a,2b,3b,4a)] (ICAPP) was custom-synthesized by Southern Research (Birmingham, AL).

ICAPP binds to the substrate-binding site of PKC-λ/ι, but not PKC-ζ [11], and has no effect on activities of recombinant forms of PKC-α, PKC-β2, PKC-δ, and PKC-ε (unpublished). Although ICAPP resembles AICAR-phosphate (the only difference being that the cyclopentane ring in ICAPP replaces the ribose ring in AICAR), ICAPP does not affect activity of adenosine monophosphate–activated protein kinase (unpublished). ICAPP potently (IC_{50}, approximately 10 nmol/L) inhibits both recombinant PKC-λ/ι activated by phosphatidylinositol-3,4,5-trisphosphate in vitro (unpublished) and insulin-stimulated aPKC activity in livers of intact mice (see below). Residual insulin-dependent hepatic aPKC activity in mouse liver that is resistant to ICAPP probably reflects PKC-ζ, which is not inhibited by ICAPP [11].

ATM binds to cysteine-69 in PKC-λ/ι and inhibits PB1 domain-dependent binding to factors that regulate downstream processes [12-16]. Although ATM does not directly inhibit activity of recombinant PKC-λ/ι or PKC-ζ, it nevertheless

diminishes insulin-induced increases in aPKC activity in intact tissues (unpublished). This suggests that PB1-dependent scaffolding is required for activation of aPKC in intact tissues. Accordingly, inhibitory effects of ATM on downstream processes in intact tissues are probably mediated by inhibition of both PB1-dependent scaffolding and enzymatic activation of aPKC.

Note that, as shown below, neither ICAPP nor ATM diminishes Akt activity or its activation by insulin.

2.2. Mouse studies

Wild-type (WT) and heterozygous muscle-specific PKC-λ knockout (Het-MλKO) mice were generated as described [10], housed in a temperature-controlled environment with alternating 12-hour light/dark cycles, and fed standard chow (ad lib feeding was continued before and throughout the experiment). All experimental procedures were approved by the Institutional Animal Care and Use Committees of the Roskamp Institute and the University of South Florida College of Medicine and by the James A. Haley Veterans Administration Medical Center Research and Development Committee, Tampa, FL.

Mice were injected subcutaneously once daily over 8 days with vehicle ± aPKC inhibitor ATM (60 mg/kg body weight) [12-15] or ICAPP (0.4 mg/kg body weight) ([11] and below). Mice were also injected intraperitoneally with vehicle ± 1 U insulin per kilogram body weight 15 minutes before killing on the eighth experimental day.

The dose of ICAPP presently used was estimated from findings (unpublished) obtained in dose-response studies conducted with both recombinant aPKCs stimulated with phosphatidylinositol-3,4,5-trisphosphate and with isolated human hepatocytes stimulated with insulin. In brief, in these ex vivo systems, ICAPP was largely effective in inhibiting PKC-λ/ι and dependent processes at approximately 10 nmol/L. The dose of ICAPP used was estimated to provide comparable nanomolar concentrations of ICAPP, assuming a uniform distribution in total body water and no turnover.

2.3. Tissue and immunoprecipitate preparations

As described [1-3,10,16], tissues were homogenized in ice-cold buffer containing 0.25 mol/L sucrose, 20 mmol/L Tris/HCl (pH 7.5), 2 mmol/L EGTA, 2 mmol/L EDTA, 1 mmol/L phenylmethylsulfonyl fluoride, 20 μg/mL leupeptin, 10 μg/mL aprotinin, 2 mmol/L $Na_4P_2O_7$, 2 mmol/L Na_3VO_4, 2 mmol/L NaF, and 1 μmol/L microcystin; supplemented with 1% Triton X-100, 0.6% Nonidet, and 150 mmol/L NaCl; and cleared by low-speed centrifugation.

2.4. aPKC assays

As described [1-3,10,16], aPKCs were immunoprecipitated from lysates with rabbit polyclonal antiserum (Santa Cruz Biotechnologies, Santa Cruz, CA) that recognizes C-termini of PKC-ζ and PKC-λ/ι. Note that PKC-ι is the human homolog of mouse PKC-λ (98% homology); human and mouse muscle contains primarily PKC-λ/ι and much lesser amounts of PKC-ζ; and mouse and human liver contains substantial, probably similar, amounts of both PKC-ζ and PKC-λ/ι [2,9].

Immunoprecipitates were collected on Sepharose-AG beads (Santa Cruz Biotechnologies) and incubated for 8 minutes at 30°C in 100-μL buffer containing 50 mmol/L Tris/HCl (pH 7.5), 100 μmol/L Na_3VO_4, 100 μmol/L $Na_4P_2O_7$, 1 mmol/L NaF, 100 μmol/L phenylmethylsulfonyl fluoride, 4 μg phosphatidylserine (Sigma, St. Louis, MO, USA), 50 μmol/L [γ-^{32}P] ATP (NEN Life Science Products, Beverly, MA), 5 mmol/L $MgCl_2$, and, as substrate, 40 μmol/L serine analogue of the PKC-ε pseudosubstrate (Millipore, Bedford, MA). After incubation, ^{32}P-labeled substrate was trapped on P-81 filter paper and counted. It is important to note that this assay reflects specific activity of a constant amount of aPKC (even in Het-MλKO mice wherein aPKC levels are diminished by approximately 40%) that is immunoprecipitated by supplier-prescribed and even considerably larger amounts of antiserum [10,16].

2.5. Western analyses

Western analyses were conducted as described [1-3,10,16] using anti-IRS-2, anti-PKC-ζ/λ, anti-p65/RelA-NFκB subunit, and anti-Akt1/2 antisera (Millipore); anti-phospho-serine-473-Akt1/2 and glyceraldehyde-phosphate dehydrogenase (GAPDH) antisera (Santa Cruz Biotechnologies); anti-PKC-ζ antiserum (gift, Dr Todd Sacktor, New York, NY); anti-p-ser-265-FoxO1 (Abnova/Taiwan, Taipei, Taiwan); and mouse monoclonal anti-PKC-ι/λ antibodies (Transduction Laboratories, Bedford, MA). Samples from experimental groups were compared on the same blots and corrected for recovery as needed by measurement of GAPDH immunoreactivity.

2.6. Messenger RNA measurements

As described [2,3,10], tissues were added to Trizol reagent (Invitrogen, Carlsbad, CA, USA); RNA was extracted and purified with RNA-Easy Mini-Kit and RNAse-Free DNAse set (Qiagen, Valencia, CA), quantified (A_{260}/A_{280}), and checked for purity by electrophoresis on 1.2% agarose gels; and messenger RNA (mRNA) was quantified by quantitative real-time reverse transcriptase-polymerase chain reaction using TaqMan reverse transcription reagent (Applied Biosystem, Carlsbad, CA). Messenger RNAs were measured with a SYBR Green kit (Applied Biosystems) using mouse nucleotide primers as follows: SREBP-1c, ATCGGCGCGGAAGCTGTCGGGGTAGCGTC (forward) and ACTGTCTTGGTTGATGAGCTGGAGCAT (reverse); FAS, GAGGACACTCAAGTGGCTGA (forward) and GTGAGGTTGCTGTCGTCTGT (reverse); ACC, GACTTCATGAATTTGCTGAT (forward) and AAGCTGAAAGCTTTCTGTCT (reverse); PEPCK, GACAGCCTGCCCCAGGCAGTGA (forward) and CTGGCCACATCTCGAGGGTCAG (reverse); G6Pase, TGCTGCTCACTTTCCCCACCAG (forward) and TCTCCAAAGTCCACAGGAGGT (reverse); IL-1β, TTGACGGACCCCAAAAGATG (forward) and AGAAGGTGCTCATGTCCTCA (reverse); TNF-α, ACGGCATGGATCTCAAAGAC (forward) and AGATAGCAAATCGGCTGACG (reverse); PKC-ζ, CATGCAGAGGCAGAGAAAACT (forward) and TTAGGTCCCGGTAGATGATCC (reverse); PKC-λ, TCACTGACTACGGCATGTGTAA (forward) and CGCAGAAAGTGCTGGTTG (reverse); and housekeeping gene hypoxanthine phosphoribosyl-transferase, TGAAAGACTTGCTCGAGATGT (forward) and AAAGAACTTATAGCCCCCCTT (reverse).

2.7. Nuclear preparations

As described [2,3,10], liver nuclei were prepared with NE-PER Nuclear and Cytoplasmic Extraction Reagents (Pierce Biotechnology, Rockford, IL).

2.8. Measurements of serum triacylglycerol, cholesterol, nonesterified fatty acids, insulin, and glucose

Serum triacylglycerol, cholesterol, nonesterified fatty acids (NEFAs), insulin, and glucose levels were measured as described [2,3,10].

2.9. Statistical evaluations

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

3. Results

3.1. Phenotype of obese Het-MλKO mice with type 2 diabetes mellitus

Partial deficiency of muscle aPKC in Het-MλKO mice produces a defect in insulin-stimulated glucose transport, specifically in muscle [2,10]. Within 5 to 6 months of age, this isolated defect in muscle leads to glucose intolerance; systemic insulin resistance; islet β-cell hyperplasia; hyperinsulinemia; increased hepatic aPKC activity; activation of hepatic SREBP-1c and inhibitor of κB kinase-β/NFκB; increased expression of hepatic SREBP-1c–dependent enzymes engaged in lipogenesis: FAS and ACC; increased NFκB-dependent expression of cytokines: TNF-α and IL-1β; increased expression of gluconeogenic enzymes: PEPCK and G6Pase; downregulation of residual aPKC activity in muscle; no significant changes in Akt activation in muscle, liver, and adipocytes; no significant changes in aPKC activation in adipocytes; hepatosteatosis; abdominal obesity; hypertriglyceridemia; hyperfattyacidemia; hypercholesterolemia; diminished glucose-lowering effects of acutely administered insulin; and mild fasting hyperglycemia ([2,10] and present results). With further aging, insulin signaling to Akt in adipose, muscle, and liver declines; insulin signaling to aPKC in adipocytes declines; and glucose intolerance progresses, as seen in 10- to 12-month-old mice used below in the ATM study (Fig. 1). Most importantly, as reported, all or most of these abnormalities in Het-MλKO mice are rapidly (within 5 days) reversed or markedly improved by administration of adenovirus encoding kinase-inactive aPKC, which selectively inhibits hepatic aPKC [2]. Accordingly, the Het-MλKO mouse provides a reliable and convenient model for testing efficacy of aPKC inhibitors and other potential therapeutic agents.

3.2. Effects of ATM treatment on insulin signaling in Het-MλKO mice

As seen in Fig. 1, insulin provoked nearly 3-fold increases in hepatic aPKC activity in WT mice (Fig. 1). However, in Het-MλKO mice, resting hepatic aPKC activity trended upward appreciably (presumably reflecting hyperinsulinemia in these ad lib–fed male mice); and insulin treatment for 15 minutes provoked further increases in aPKC activity that exceeded those seen in insulin-treated WT mice (Fig. 1). Insulin also provoked increases in hepatic Akt activity, but these increases were similar in WT and Het-MλKO mice (Fig. 1).

Following treatment of Het-MλKO mice with ATM for 7 days, both resting and insulin-stimulated hepatic aPKC activities diminished by 50% or more (Fig. 1). In contrast to these decreases in aPKC activation, insulin-stimulated phosphorylation of hepatic Akt (presumably reflective of activation) trended upward after ATM treatment (Fig. 1).

As expected, insulin increased activity of aPKC in adipose tissue and muscle tissues of WT mice (Fig. 1). However, in Het-MλKO mice, unlike the situation in liver, where resting and insulin-stimulated aPKC activities were elevated, insulin failed to significantly increase activity of either the full complement of aPKC in adipose tissue or the residual aPKC in muscle (Fig. 1). It was therefore interesting to find that ATM treatment improved resting and/or insulin-stimulated aPKC activity in both adipose and muscle tissues of Het-MλKO mice (Fig. 1). In this regard, note that, as stated in "Methods," the aPKC assay reflects specific enzyme activity of aPKC rather than the total amount of aPKC in the tissue. Also note that the diminished levels of aPKC present in muscles of Het-MλKO mice [10] were not altered by ATM or ICAPP treatments. Thus, it is likely that a deficiency of total aPKC activity (which would not reflected by the aPKC assay) would still be present in muscles of ATM-treated Het-MλKO mice.

It was similarly interesting to find that, as with aPKC activity, resting and/or insulin-stimulated Akt phosphorylation in muscle and adipose tissues of vehicle-treated Het-MλKO mice was diminished; and more importantly, Akt phosphorylation in both tissues increased or trended upward after ATM treatment (Fig. 1).

3.3. Effects of ATM treatment on serum insulin and glucose levels of Het-MλKO mice

In association with impaired activation of muscle aPKC and Akt in Het-MλKO mice, resting (ie, nonfasting, ad lib fed) serum insulin levels were elevated; and ability of insulin to acutely (over 15 minutes) diminish serum glucose levels was impaired (Fig. 2). However, following ATM treatment, resting serum insulin levels and ability of insulin to acutely decrease serum glucose levels of Het-MλKO mice were comparable to those of WT mice (Fig. 2).

3.4. Effects of ATM treatment on hepatic enzymes of Het-MλKO mice

In livers of Het-MλKO mice, resting (ie, nonfasting, ad lib fed) mRNA levels of (*a*) lipogenic factors SREBP-1c, FAS, and ACC and (*b*) gluconeogenic factors PEPCK and G6Pase, and (*c*) tissue levels of triacylglycerols were elevated (Fig. 3). In association with decreases in hepatic aPKC activity in ATM-treated Het-MλKO mice, expression of hepatic SREBP-1c, FAS, ACC, PEPCK, and G6Pase, and levels of hepatic triacylglycerol diminished to or toward WT levels (Fig. 3).

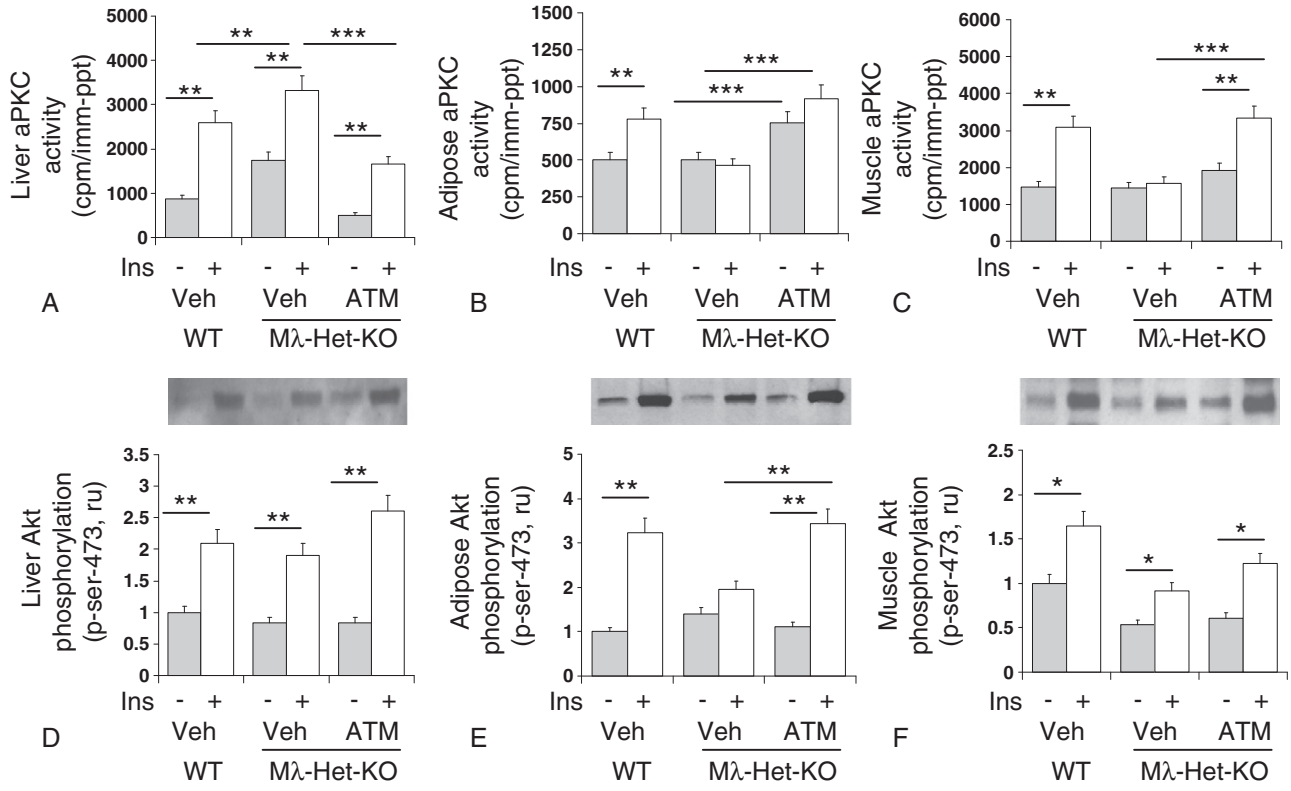

Fig. 1 – Effects of treatment of Het-MλKO mice with aPKC inhibitor ATM on resting (nonfasting) and insulin-stimulated activities of aPKC (A, B, and C) and ser-473 phosphorylation of Akt2 (C, D, and E) in liver (A and D), adipose tissue (B and E), and muscle (C and F). Ad lib–fed male Het-MλKO mice (10-12 months of age) were treated with vehicle (Veh) or ATM in vehicle (60 mg/kg body weight per day) for 7 days; and on the eighth day, the Het-MλKO mice, along with littermate WT mice, were treated for 15 minutes by intraperitoneal injection of vehicle alone (–) or vehicle containing insulin (1 U/kg body weight) (+), and then killed. Bar gram values are mean ± SEM of 6 mice. Asterisks (*P < .05; **P < .01; ***P < .001) reflect *P* values of indicated groups. Representative immunoblots for p-ser-473-Akt are shown above bar grams.

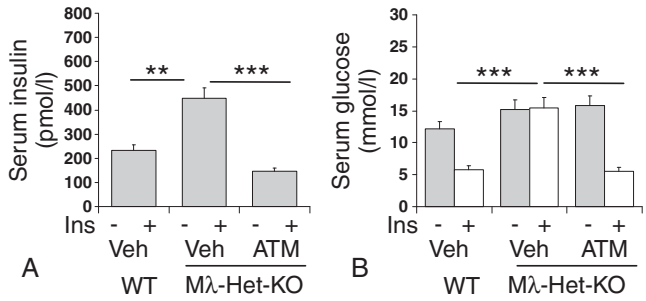

Fig. 2 – Effects of treatment of Het-MλKO mice with aPKC inhibitor ATM on resting (nonfasting) insulin levels (A) and resting (nonfasting) and insulin-stimulated serum levels of glucose (B). Ad lib–fed male Het-MλKO mice (10-12 months of age) were treated with vehicle (Veh) or ATM in vehicle (60 mg/kg body weight per day) for 7 days; and on the eighth day, these Het-MλKO mice, along with littermate WT mice, were treated for 15 minutes by intraperitoneal injection of vehicle alone (–) or vehicle containing insulin (1 U/kg body weight) (+), and then killed. Bar gram values are mean ± SEM of 6 mice. Asterisks (*P < .05; **P < .01; ***P < .001) reflect *P* values of indicated groups. Data are from mice used in the study described in Fig. 1.

In addition to ATM-induced alterations in hepatic lipogenic and gluconeogenic pathways, active nuclear levels of the hepatic NFκB/p65/RelA subunit were diminished by ATM treatment (Fig. 3). This decrease in NFκB activation was accompanied by significant decreases to WT levels ($P < .05$) in mRNA levels of hepatic proinflammatory cytokines TNF-α and IL-1β, which were increased 1.6- to 3-fold in livers of vehicle-treated Het-MλKO mice (data not shown).

3.5. *Effects of ATM treatment on obesity and lipid abnormalities in Het-MλKO mice*

As seen in Fig. 3, abdominal fat content (combined omental, retroperitoneal, and perigonadal fat) and serum levels of triacylglycerol and cholesterol were elevated in vehicle-treated Het-MλKO mice. In conjunction with ATM-induced improvements in hepatic expression of lipogenic factors in Het-MλKO mice, abdominal fat content and serum levels of triacylglycerol and cholesterol returned to levels comparable to those seen in WT mice (Fig. 3).

Germane to improvement in abdominal obesity seen in Het-MλKO mice, 7-day food intake trended downward by 21% (0.61 ± 0.06 [mean ± SEM; n = 12] in vehicle-treated vs 0.48 ± 0.03 [n = 12] g food per gram body weight) ($P < .07$).

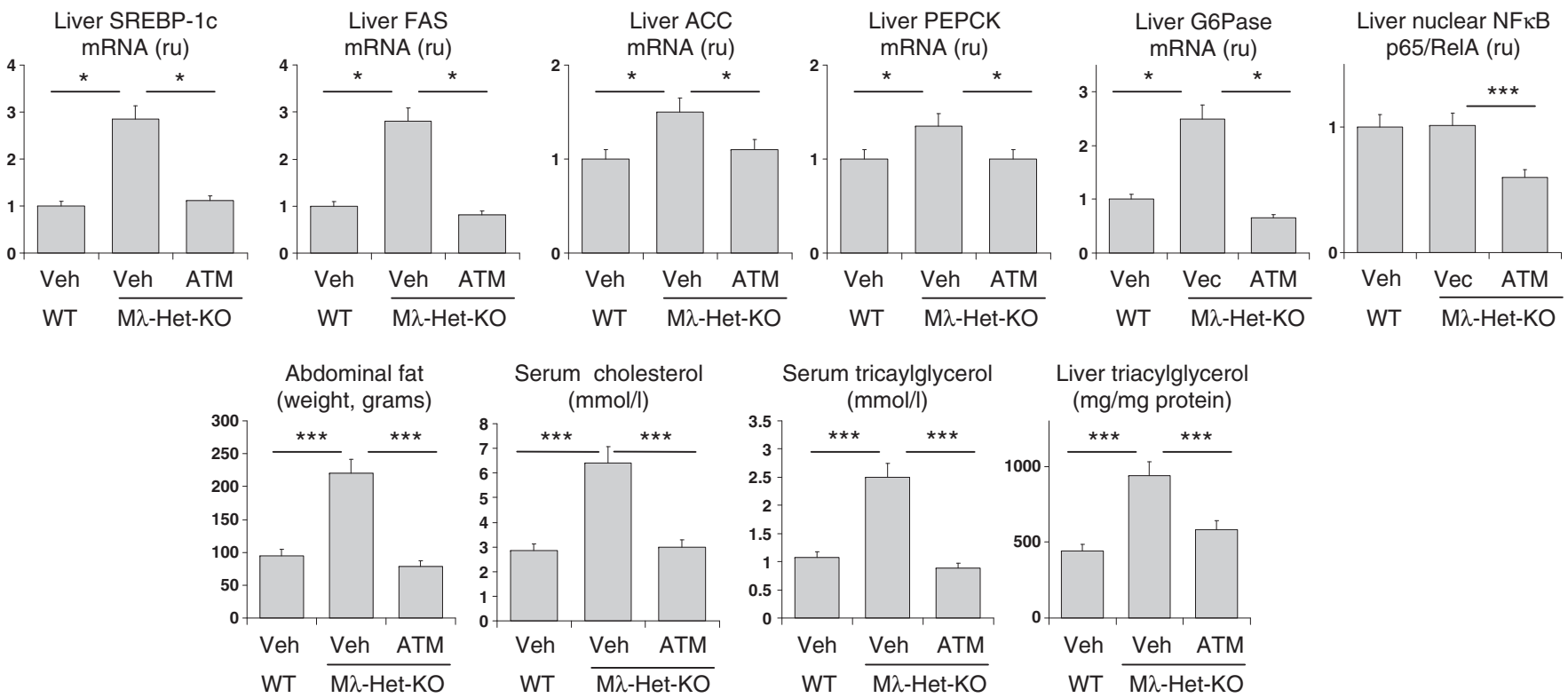

Fig. 3 – Effects of treatment of Het-MλKO mice with aPKC inhibitor ATM on expression of lipogenic (SREBP-1c, FAS, ACC) and gluconeogenic (PEPCK, G6Pase) enzymes and nuclear levels of the active NFκB/p65/RelA subunit in liver (top row), and clinical parameters, namely, abdominal (combined omental, retroperitoneal, and perigonadal) fat weight, serum levels of triacylglycerol and cholesterol, and hepatic levels of triacylglycerol (bottom row). Ad lib–fed male Het-MλKO mice (10-12 months of age) were treated with vehicle (Veh) or ATM in vehicle (60 mg/kg body weight per day) for 7 days; and on the eighth day, the Het-MλKO mice, along with littermate WT mice, were treated for 15 minutes by intraperitoneal injection with vehicle alone (–) or with vehicle containing insulin (1 U/kg body weight) (+), and then killed (note that acute insulin treatment did not alter hepatic enzyme expression or serum lipid levels). Values are mean ± SEM of 12 mice. RU indicates relative units. *P* values (*P < .05; **P < .01; ***P < .001; ANOVA) reflect comparisons between indicated groups. Data are from mice used in the study described in Fig. 1 (note that these parameters were not altered by acute 15-minute insulin treatment).

However, note that food intake was increased by 20% in untreated Het-MλKO mice [10] and that food intake was not altered in WT mice treated with ATM for 6 weeks (data not shown).

Of further note, glucose tolerance and insulin released during a 2-hour glucose tolerance test were unaltered in WT mice treated with ATM for 6 weeks (data not shown).

3.6. *Effects of ICAPP treatment on insulin signaling in Het-MλKO mice*

Similar to findings in the above-described ATM study involving 10- to 12-month-old male Het-MλKO mice, resting and insulin-stimulated hepatic aPKC activities were elevated in vehicle-treated Het-MλKO mice used in the ICAPP study wherein 5- to 7-month-old, ad lib–fed female Het-MλKO mice were used (Fig. 4). In contrast to hepatic aPKC, resting and insulin-stimulated activities of aPKC were diminished or trended downward in muscle and adipose tissues of vehicle-treated Het-MλKO mice (Fig. 4).

Partly dissimilar from findings in the ATM study, Akt activation in liver, muscle, and adipose tissue showed upward trends or was comparable to activities in WT mice in the ICAPP study (Fig. 4). These differences in Akt activation in the ATM and ICAPP studies may reflect differences in age and/or sex of Het-MλKO mice used in these studies. In this regard, note that female Het-MλKO mice are less glucose intolerant than male Het-MλKO mice [10], perhaps reflecting better preservation of Akt signaling; and Akt activation in all tissues is fully intact in 4- to 6-month-old Het-MλKO mice [10] but, as seen here in the ATM study, may diminish in conjunction with age-related increases in glucose intolerance.

As with ATM treatment, 7-day treatment of Het-MλKO mice with ICAPP substantially diminished insulin-stimulated activity of hepatic aPKC activity; but, opposite to aPKC, hepatic Akt phosphorylation trended further upward (Fig. 4). Similarly, resting and/or insulin-stimulated activities of aPKC and phosphorylation of Akt in adipose and muscle tissues were increased or trended upward following ICAPP treatment of Het-MλKO mice (Fig. 4).

3.7. *Effects of ICAPP treatment on serum insulin and glucose levels in Het-MλKO mice*

In conjunction with improvements in insulin signaling in muscle and adipose tissues and as found in the ATM study, resting serum insulin levels, resting serum glucose levels, and serum glucose levels following acute 15-minute insulin

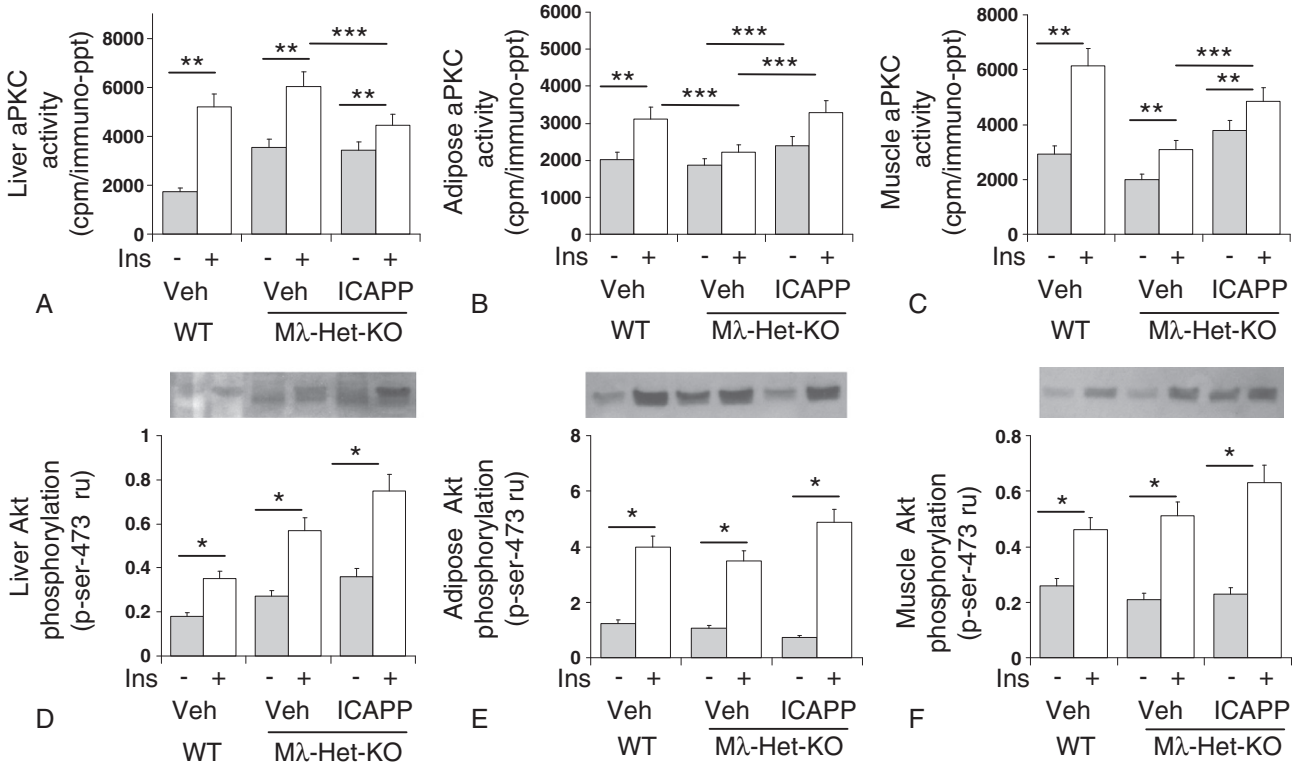

Fig. 4 – Effects of treatment of Het-MλKO mice with aPKC inhibitor ICAPP on resting (nonfasting) and insulin-stimulated activity of aPKC (A, B, and C) and ser-473 phosphorylation of Akt2 (E, F, and G) in liver (A and D), adipose tissue (B and E), and muscle (C and F). Ad lib–fed female Het-MλKO mice (5-7 months of age) were treated with vehicle (Veh) or ICAPP in vehicle (0.4 mg/kg body weight per day) for 7 days; and on the eighth day, the Het-MλKO mice and littermate WT mice were treated for 15 minutes by intraperitoneal injection of vehicle alone (–) or vehicle containing insulin (1 U/kg body weight) (+), and then killed. Values are mean ± SEM of 6 mice. Asterisks (*P < .05; **P < .01; ***P < .001) reflect *P* values of indicated groups. Representative immunoblots for p-ser-473-Akt are shown above bar grams.

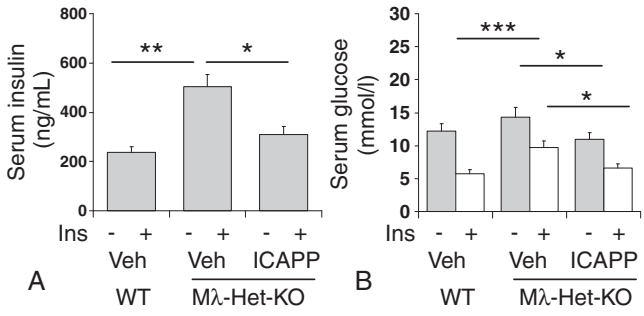

Fig. 5 – Effects of treatment of Het-MλKO mice with aPKC inhibitor ICAPP on resting (nonfasting) insulin levels (A) and resting (nonfasting) and insulin-stimulated serum levels of glucose (B). Ad lib fed–female Het-MλKO mice (5-7 months of age) were treated with vehicle (Veh) or ICAPP in vehicle (0.4 mg/kg body weight per day) for 7 days; and on the eighth day, the Het-MλKO mice, along with littermate WT mice, were treated for 15 minutes by intraperitoneal injection of vehicle alone (–) or vehicle containing insulin (1 U/kg body weight) (+), and then killed. Bar gram values are mean ± SEM of 6 mice. Asterisks (*P < .05; **P < .01; ***P < .001) reflect P values of indicated groups. Data are from mice used in the study described in Fig. 4.

treatment diminished to or toward WT levels in ICAPP-treated Het-MλKO mice (Fig. 5).

3.8. *Effects of ICAPP treatment on hepatic enzymes in Het-MλKO mice*

Similar to findings in the ATM study, mRNA levels for hepatic lipogenic factors SREBP-1c, FAS, and ACC, and mRNA levels for gluconeogenic factors PEPCK and G6Pase in ICAPP-treated Het-MλKO mice diminished to or toward WT levels following ICAPP treatment in Het-MλKO mice (Fig. 6).

3.9. *Effects of ICAPP treatment on obesity and lipid abnormalities in Het-MλKO mice*

In association with improvements in hepatic lipogenic and gluconeogenic enzyme expression following ICAPP treatment, the increases in abdominal fat content, hepatic triacylglycerol content, and serum levels of triacylglycerol, cholesterol, and NEFAs in seen in untreated Het-MλKO mice diminished to or toward WT levels (Fig. 6). In this regard, note that food intake over the 7-day period was not altered by ICAPP treatment (0.95 ± 0.09 [mean ± SEM; n = 12] in untreated vs 1.09 ± 0.09 [n = 12] g food per gram body weight) in ICAPP-treated mice).

3.10. *Selective effects of ICAPP on aPKC activity in liver*

It was surprising to find in the above-described studies that muscle and adipose tissue aPKC activities of Het-MλKO mice increased following ICAPP and ATM treatments. To gain further insight into this finding, we examined the effective tissue distribution of ICAPP after subcutaneous administration in WT mice (which obviously have full complements of aPKCs in all tissues) by measuring basal and insulin-stimulated aPKC activities in liver, muscle, and adipose tissues. In keeping with findings observed in studies of Het-MλKO mice, ICAPP diminished insulin-stimulated aPKC activity in liver, but not in either muscle or adipose tissues of WT mice (Fig. 7). Also note that (*a*) ser-256 phosphorylation of hepatic FoxO1 was increased by ICAPP both basally and in response to insulin, reflecting intact or heightened hepatic Akt activation, and (*b*) inhibition of hepatic aPKC and stimulatory effects on FoxO1 phosphorylation persisted unchanged for at least 30 hours after a single injection of ICAPP (1.5 mg/kg body weight) (Fig. 7).

4. Discussion

As in previous studies [2,10], we found that hepatic PKC-λ in ad lib–fed Het-MλKO mice is maintained in an overactive state and contributes importantly to excessive activation of hepatic lipogenic, proinflammatory, and gluconeogenic pathways that, in turn, contribute importantly in the pathogenesis of clinical lipid and carbohydrate abnormalities in Het-MλKO mice. Accordingly, the Het-MλKO mouse appears to be well suited to serve as a model of obesity, with associated metabolic syndrome features, and early type 2 diabetes mellitus. Moreover, the rapidity and completeness of responsiveness of virtually all metabolic abnormalities in Het-MλKO mice noted previously in adenoviral-mediated hepatic expression studies [2] and noted here are particularly helpful for testing therapeutic interventions for these disorders.

Accordingly, we used the Het-MλKO mouse model to determine the efficacy of 2 recently developed small-molecule inhibitors of PKC-λ for their ability to inhibit insulin signaling to aPKC in liver and thereby diminish aPKC-dependent regulation of hepatic factors that contribute importantly to development of clinical lipid and carbohydrate abnormalities in obesity, the metabolic syndrome, and type 2 diabetes mellitus.

It was of course surprising to find that both ATM and ICAPP treatments in vivo selectively inhibited hepatic aPKC but spared aPKC in muscle and adipose tissues of Het-MλKO. In fact, insulin signaling to aPKC, as well as Akt, increased in both muscle and adipose tissues of Het-MλKO mice after ATM and ICAPP treatments.

The reason for selective impairment of hepatic aPKC and sparing of muscle and adipose tissue aPKC during in vivo treatment with ICAPP and ATM is presently uncertain. This selectivity does not appear to be due to inability of ICAPP to penetrate these cell types, as ICAPP inhibited insulin stimulation of aPKC activity and glucose transport in PKC-λ–rich isolated mouse adipocytes and cultured 3T3/L1 adipocytes and, similarly, ATM inhibited insulin-stimulated aPKC activity and glucose transport in PKC-ζ–rich cultured L6 myotubes (unpublished).

On the other hand, when a relatively high dose of ICAPP (three times that used to effectively inhibit hepatic PKC-ι in the ICAPP study) was administered to WT mice, insulin-stimulated aPKC activity diminished in liver, but not in muscle or adipose tissue. This preferential inhibition of hepatic aPKC in vivo may partly reflect that aPKC levels are much higher in liver than in muscle and adipose tissue (unpublished), but

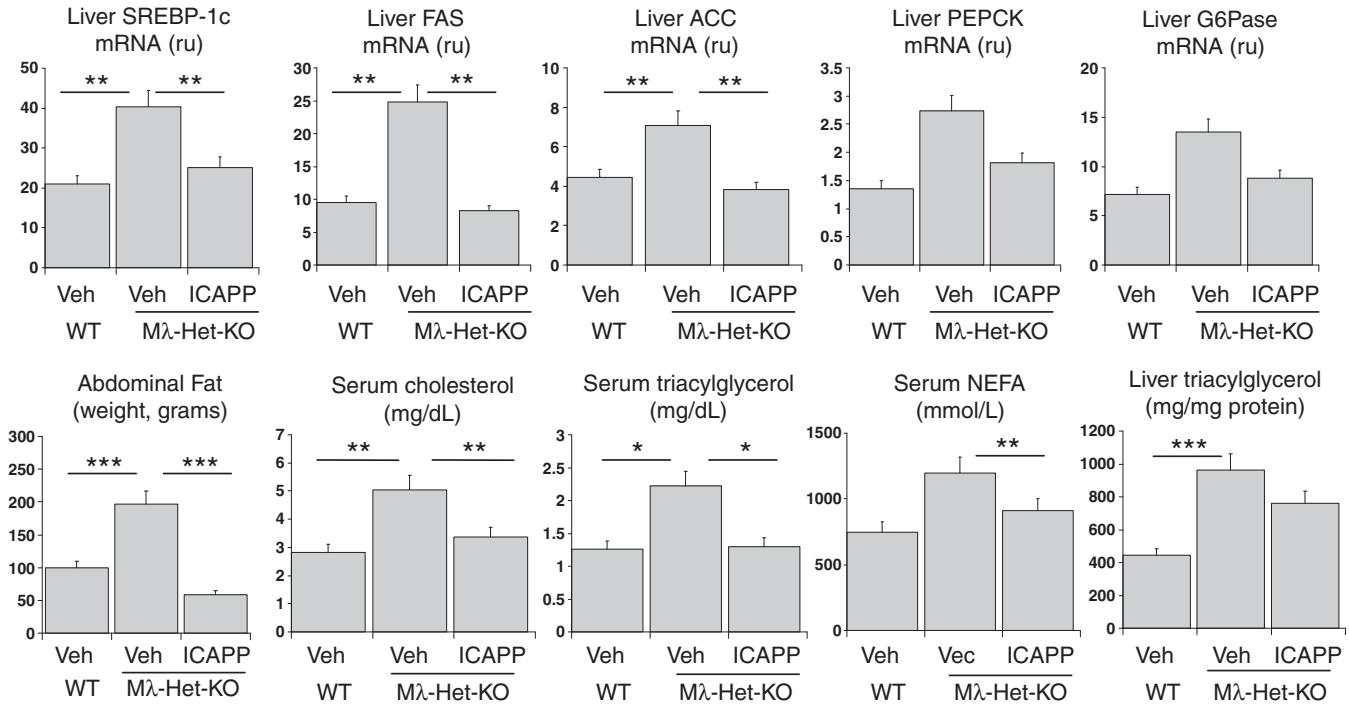

Fig. 6 – Effects of treatment of Het-MλKO mice with aPKC inhibitor ICAPP on expression of lipogenic (SREBP-1c, FAS, ACC) and gluconeogenic (PEPCK, G6Pase) enzymes and nuclear levels of the active NFκB/p65/RelA subunit in liver (top row), and clinical parameters, namely, abdominal (combined omental, retroperitoneal, and perigonadal) fat weight, serum levels of triacylglycerol and cholesterol, and hepatic levels of triacylglycerol (bottom row). Ad lib–fed female Het-MλKO mice (5-7 months of age) were treated with vehicle (Veh) or ICAPPM in vehicle (0.4 mg/kg body weight per day) for 7 days; and on the eighth day, the Het-MλKO mice, along with littermate WT mice, were treated for 15 minutes by intraperitoneal injection with vehicle alone (–) or with vehicle containing insulin (1 U/kg body weight) (+), and then killed (note that acute insulin treatment did not alter hepatic enzyme expression or serum lipid levels). Values are mean ± SEM of 6 to 9 mice in the top row and 12 mice in the bottom row. RU indicates relative units. *P* values (P* < .05; ***P* < .01; ****P* < .001; ANOVA) reflect comparisons between indicated groups. Data are from mice used in the study described in Fig. 4 (note that these parameters were not altered by acute 15-minute insulin treatment).**

other reasons must also be considered. For example, blood flow and clearance of ICAPP and ATM may be greater in liver than in muscle or adipose tissues. Also note that activation of aPKC involves molecular unfolding and opening of the substrate-binding site [8], and this activation process may facilitate ICAPP access to the substrate-binding site. Accordingly, because hepatic aPKC is hyperactive in Het-MλKO mice (as opposed to hypoactive aPKC in muscle and adipose tissue), this increase in hepatic activity may favor hepatic partitioning of ICAPP (analogously, opening of the cysteine-69 residue in the PB1-binding domain site may enable ATM access). Finally, in addition to potent binding of ICAPP PKC-ι, the prolonged inhibition of hepatic PKC-ι noted presently suggests that this binding is tight; thus, the retention of ICAPP on active hepatic enzyme may also favor hepatic partitioning.

Improved glucose-lowering effects of acute insulin treatment in Het-MλKO mice following ATM and ICAPP treatment seem likely to be reflective of improved insulin signaling and glucose uptake in muscle and adipose tissues. Although this rapid glucose-lowering effect of insulin was unattended by alterations in expression of hepatic enzymes, a contribution of the liver cannot be ruled out. On the other hand, ATM- and ICAPP-induced decreases in hepatic gluconeogenic enzymes PEPCK and G6Pase are likely to have contributed importantly to improvements in overall glucose homeostasis in Het-MλKO mice. Indeed, the expression of gluconeogenic PEPCK and G6Pase that was increased in the ad lib–fed Het-MλKO mice most likely contributed to glucose intolerance in untreated Het-MλKO mice. It is uncertain if these improvements in glucose homeostasis in hepatic and extrahepatic tissues reflected alterations in circulating lipids, cytokines, or other factors that are thought to influence systemic insulin resistance.

It may be recalled that fasting glucose levels, as well as post–glucose loading serum glucose levels, were found to be modestly but significantly increased in Het-MλKO [10]. Although clamp studies showed only slight trends for increases in hepatic glucose output in Het-MλKO and homozygous-MλKO mice, these clamp studies were performed in mice that had been recently undergone preclamp operative procedures; and fasting serum glucose were not elevated in these mice, perhaps reflecting favorable alterations in basal status of these knockout mice. In any case, the fact that ad lib–fed Het-MλKO mice have elevated expression of hepatic gluconeogenic enzymes, as well as fasting hyperglycemia, raises

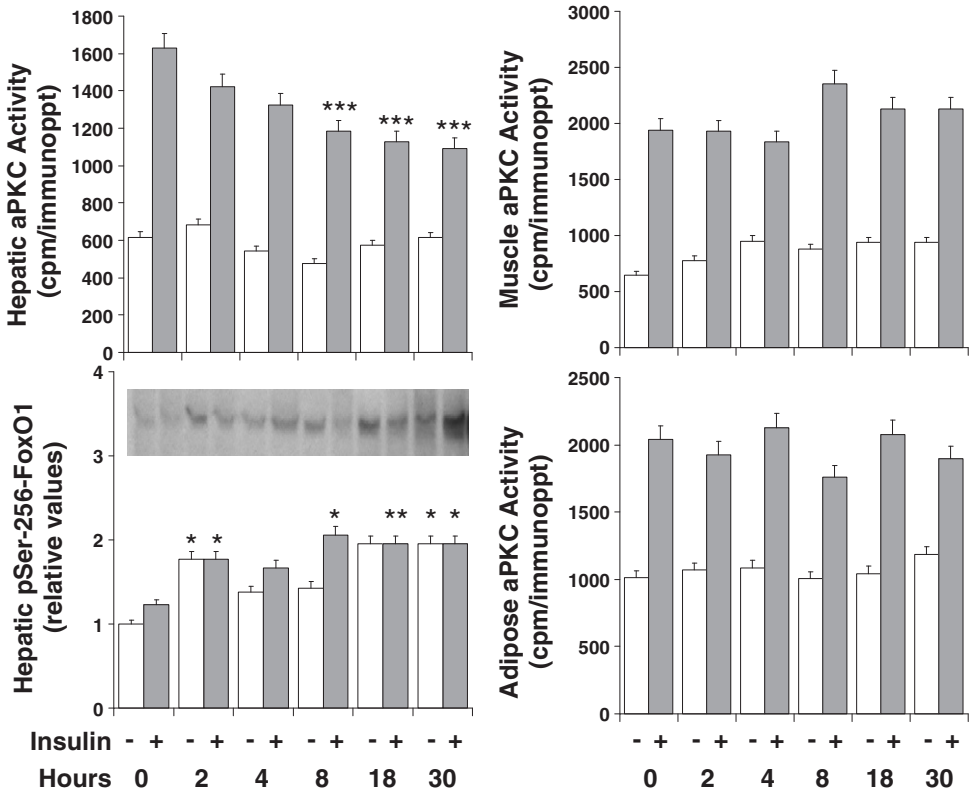

Fig. 7 – Selective inhibition of hepatic aPKC during ICAPP treatment in vivo. ICAPP (1.5 mg/kg body weight) was administered subcutaneously to WT mice; and after the indicated periods, mice were treated with or without insulin (1 U/kg body weight) 15 minutes before killing. Alterations in basal and insulin-stimulated aPKC activity were examined in liver, muscle, and adipose tissues, and on p-Ser-256-FoxO1 in liver. Values are mean ± SEM of 6 to 8 determinations. *P < .05; **P < .01; ***P < .001; ANOVA. Note that the single dose of ICAPP used in this experiment was 3 times greater than that used in Figs 4, 5, and 6. Also note prolonged effects of ICAPP on hepatic aPKC activity.

the possibility that the liver may have contributed importantly to the glucose intolerance originally observed [10] in Het-MλKO mice.

The improvement in abdominal obesity in Het-MλKO mice with ATM and ICAPP treatment most likely reflects decreases in hepatic lipid synthesis, coupled perhaps with small difficult-to-measure decreases in food intake. In this regard, it is interesting that the 21% downward trend in food intake during ATM treatment closely matches the 20% increase food intake observed in untreated Het-MλKO mice [10]. This matchup raises the possibility that liver-derived, aPKC-dependent lipids, cytokines, and/or other factors in Het-MλKO mice may have inhibited central nervous system (CNS) appetite-suppression mechanisms; and this CNS appetite-suppression may have been reversed by inhibition of hepatic aPKC. (Note that it is unlikely that diminished food intake during ATM treatment reflected direct effects of ATM on CNS centers involved in appetite suppression, as aPKC inhibition in these centers diminishes appetite-suppressing effects of certain agents [17].) On the other hand, we did not observe any decreases in food intake in ICAPP-treated mice; and the improvement in abdominal obesity in these mice may have simply reflected a reduced capacity of the liver to produce circulating lipids that are used for storage in adipose depots.

It may be recalled that total aPKC levels are diminished by approximately 40% and total aPKC activity is diminished by approximately 70% in muscles of humans with type 2 diabetes mellitus [11,18]. More recently, we found that the 40% decrease in total aPKC levels is largely accounted for by 50% deficiencies of both protein and mRNA levels of PKC-ι, which is much more abundant than PKC-ζ in human muscle (unpublished). This deficiency of PKC-ι in muscles of humans with type 2 diabetes mellitus is in fact similar to the gene-knockout–induced deficiency of PKC-λ in muscles of presently used Het-MλKO mice. Thus, deficiency of muscle aPKC in Het-MλKO mice can be considered as a human-diabetes–like feature. However, this does not imply that diminished expression of muscle PKC-ι in human muscle is due to a comparable genetic alteration. At present, the cause for this defect is uncertain.

As another human-diabetes–like feature of Het-MλKO mice, we have also recently found that, opposite to muscle, hepatic PKC-ι is overactive in humans with type 2 diabetes mellitus (unpublished). In short, there is marked imbalance between underactive aPKC in muscle and overactive aPKC in liver in both Het-MλKO mice and humans with type 2 diabetes mellitus. In this regard, it is important to note that this imbalance is particularly problematic. Thus, deficient PKC-λ/ι and activation and glucose transport in muscle increase glucose

intolerance and insulin resistance; and resultant hyperinsulinemia increases activity of hepatic PKC-λ/ι and expression of hepatic lipogenic, proinflammatory, and gluconeogenic pathways. These hepatic alterations, in turn, contribute to further increases in insulin resistance; further impairment of insulin signaling in liver, muscle, and adipocytes; further increases in hyperglycemia and hyperinsulinemia; and further activation of hepatic aPKC, which is downstream of IRS-2 in the liver and which is well maintained even as diabetes progresses [1]. Fortunately, as seen herein, this vicious cycle can be overcome by inhibitors of hepatic PKC-ι.

Finally, the present studies demonstrate that small-molecular, highly specific inhibitors of PKC-λ/ι, such ICAPP and ATM, can be used to selectively target hepatic PKC-λ/ι in an in vivo setting and markedly improve obesity, hyperlipidemia, and glucose metabolism in a mouse model of obesity, the metabolic syndrome, and type 2 diabetes mellitus. Accordingly, it is reasonable to suggest that similar selectivity and efficacy will be seen in other species, including humans who have obesity, the metabolic syndrome, and/or type 2 diabetes mellitus. Clearly, there is a pressing need for further studies.

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Conflict of Interest

No conflicts of interest.

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