

# Lipid Mediators of Insulin Resistance

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*Lipid abnormalities such as obesity, increased circulating free fatty acid levels, and excess intramyocellular lipid accumulation are frequently associated with insulin resistance. These observations have prompted investigators to speculate that the accumulation of lipids in tissues not suited for fat storage (e.g., skeletal muscle and liver) is an underlying component of insulin resistance and the metabolic syndrome. We review the metabolic fates of lipids in insulin-responsive tissues and discuss the roles of specific lipid metabolites (e.g., ceramides, GM3 ganglioside, and diacylglycerol) as antagonists of insulin signaling and action.*

**Key words:** insulin resistance, lipid, mediators, obesity

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doi: 10.1301/nr.2007.jun.S39–S46

## INTRODUCTION

The peptide hormone insulin stimulates the uptake and storage of glucose in skeletal muscle and adipose tissue while simultaneously inhibiting its efflux from the liver. In certain pathological conditions, including type 2 diabetes mellitus and the metabolic syndrome, these tissues become resistant to insulin such that a physiological dose of the hormone is unable to elicit these anabolic responses. Numerous studies suggest that the oversupply of lipid to peripheral tissues might contribute to the development of this insulin resistance.<sup>1</sup> First, insulin-resistant subjects frequently display signs of abnormal lipid metabolism, including obesity, increased circulating free fatty acid (FFA) concentrations, and elevated intramyocellular lipid levels. Second, experimentally ex-

posing peripheral tissues to lipids decreases their sensitivity to insulin. For example, incubating isolated muscle strips or cultured muscle cells with FFAs, infusing lipid emulsions into rodents or humans, or expressing lipoprotein lipase in skeletal muscle of transgenic mice promotes intramyocellular lipid accumulation and compromises insulin-stimulated glucose uptake. These observations have prompted investigators to hypothesize that increased availability of lipids to peripheral tissues induces insulin resistance by promoting the accumulation of one or more fat-derived metabolites capable of inhibiting insulin action.

## INSULIN SIGNALING AND ACTION

Insulin accelerates glucose entry into skeletal muscle and adipose tissue by evoking the translocation of GLUT4 glucose transporters from intracellular stores to the plasma membrane. Simultaneously, the hormone regulates numerous metabolic enzymes (e.g., glycogen synthase or pp70 S6-kinase) to promote storage of the incoming glucose as glycogen, triglyceride, or protein. Insulin initiates these pleiotropic actions through its heterotetrameric receptor with intrinsic tyrosine kinase activity (Figure 1). The activated receptor phosphorylates a family of insulin receptor substrates (IRS proteins) that recruit and activate intracellular effector enzymes. One of these docking proteins, the lipid kinase phosphatidylinositol 3-kinase (PI3K), is a requisite intermediate in insulin's metabolic, anti-apoptotic, and mitogenic effects. PI3K initiates a widely conserved signaling pathway leading to the activation of Akt/protein kinase B (PKB), which is required for insulin-stimulated glucose uptake and anabolic metabolism. In the following sections, we will discuss the capacity of several lipid intermediates to inactivate components of this insulin signaling pathway.

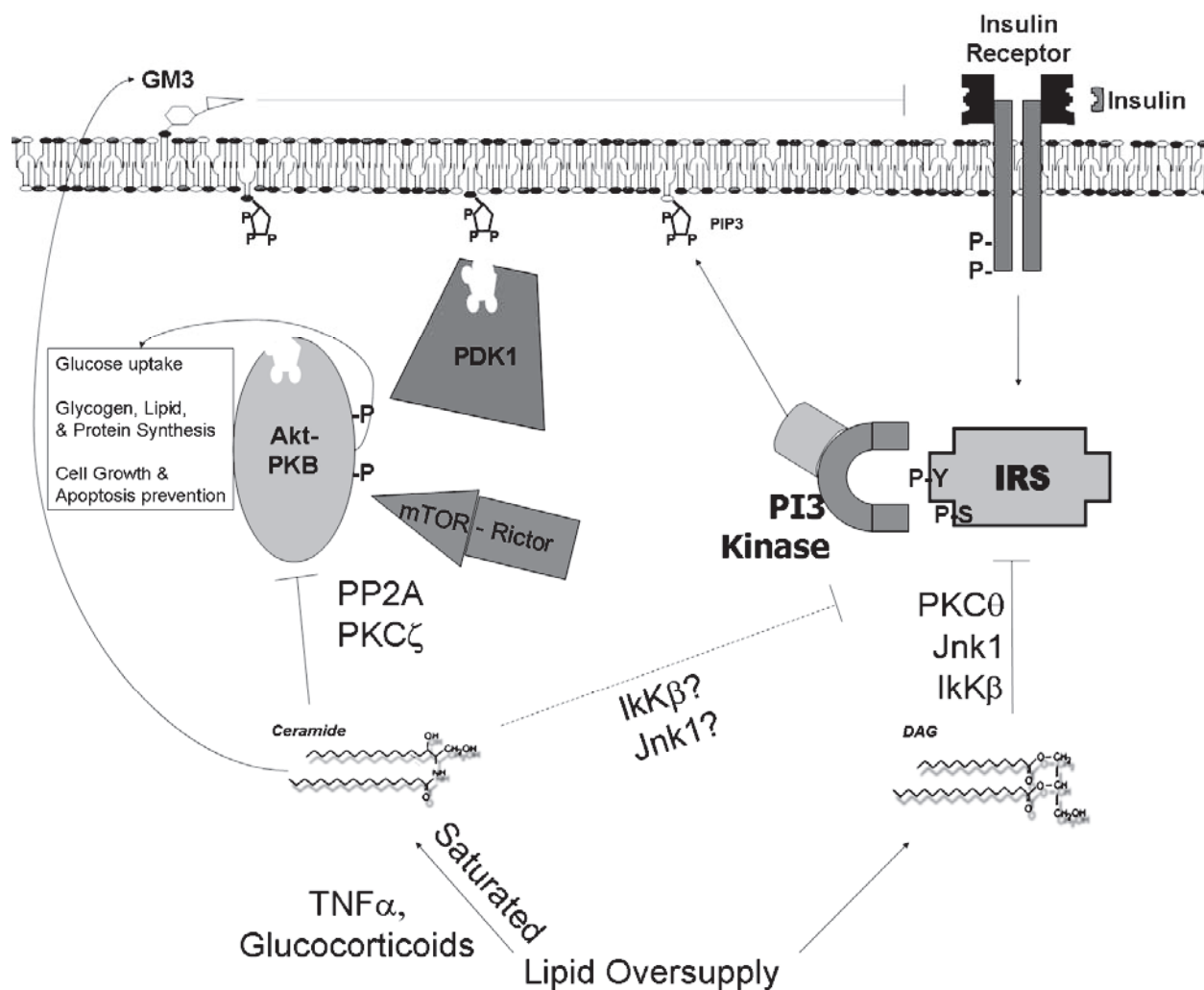
## INTRACELLULAR LIPID METABOLITES IN THE INDUCTION OF INSULIN RESISTANCE

Lipoprotein lipases hydrolyze triglycerides present in circulating chylomicrons or very low density lipopro-

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**Figure 1.** Schematic depicting the insulin signaling cascade and the antagonistic effects of lipid metabolites. DAG, diacylglycerol; GM3, GM3 ganglioside; IκKβ, inhibitor of κB-kinase-β; IRS, insulin receptor substrates; Jnk1, c-jun-N-terminal kinase 1; mTOR, mammalian target of rapamycin; PDK1, phosphatidylinositol-dependent kinase 1; PI3-kinase, phosphatidylinositol 3-kinase; PIP3, phosphatidylinositol 3-5 trisphosphate; PKB, protein kinase B; PKC, protein kinase C; PP2A, protein phosphatase 2A; TNFα, tumor necrosis factor alpha.

teins (VLDLs), liberating FFAs, which traverse the plasma membrane by diffusion or with the assistance of putative fatty acid transport proteins such as CD36, very long chain acyl-CoA synthetases, or caveolin 1.<sup>2</sup> Acyl-CoA synthetases (ACS) add a CoA thioester to fatty acids, trapping them within the cell and preparing them for subsequent metabolic fates.

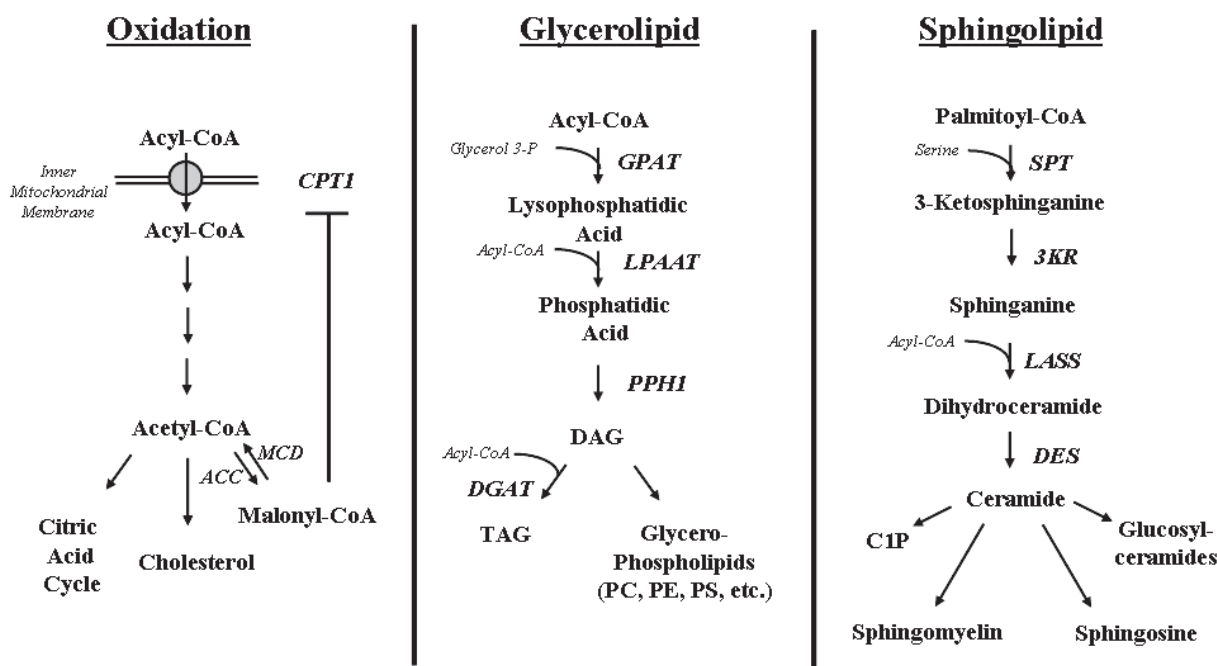
### Fates of Intracellular Lipids

Fatty-acyl CoAs, depending on their composition and the energy needs of the cell, may be shunted into one of three competing pathways: beta-oxidation, glycerolipid formation, or sphingolipid formation (Figure 2).

Acyl-CoAs can undergo beta-oxidation within the mitochondria, allowing energy equivalents to be donated to the electron transport chain for subsequent generation

of ATP. The rate of lipid oxidation is largely governed by carnitine palmitoyltransferase I (CPT I), which regulates mitochondrial entry of long-chain fatty acyl-CoAs. Short-chain fatty acids can bypass the carnitine shuttle system consisting of CPT I and CPT II at the outer and inner mitochondrial membranes, respectively, and thus are unlikely to contribute to the formation of other lipid metabolites. A key allosteric inhibitor of CPTI is malonyl-CoA, which is produced from acetyl-CoA in a reaction catalyzed by acetyl-CoA carboxylase (ACC). Because oxidation of carbohydrates also leads to an increase in acetyl-CoA levels, this regulatory event allows for cross talk between the catabolic pathways controlling lipid and glucose metabolism, and provides a mechanism for blocking lipid oxidation when cellular energy needs are low.

The majority of fatty acids are incorporated into



**Figure 2.** Schematic depicting the metabolic fates of intracellular fatty acids. CPT1, carnitine palmitoyltransferase; DAG, diacylglycerol; DES, dihydroceramide desaturase; DGAT, diacylglycerol acyltransferase; GPAT, glycerol-3-phosphate acyltransferase; 3KR, 3-ketosphinganine reductase; LASS, longevity assurance genes; LPAAT, lysophosphatidic acid acyltransferase; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PPH1, phosphatidate phosphohydrolase; PS, phosphatidylserine; SPT, serine palmitoyltransferase; TAG, triacylglycerol.

glycerolipids, including membrane glycerophospholipids and di- or triacylglycerols. The initial committed step in glycerolipid synthesis is the acylation of sn-glycerol-3-phosphate to form lysophosphatidic acid by glycerol-3-phosphate acyltransferase (GPAT). Subsequent acylation of LPA leads to the production of phosphatidic acid, which is dephosphorylated to produce the parent molecule diacylglycerol (DAG). DAG can be further metabolized into triglyceride, which is the primary form of stored fat in adipocytes, or into glycerophospholipids (e.g., phosphatidic acid, phosphatidylcholine, etc.). While these phospholipid molecules have not been implicated in insulin resistance, a number of less abundant glycerolipids (discussed below) have been identified as potential intermediates linking lipid oversupply to the antagonism of insulin signaling or action.

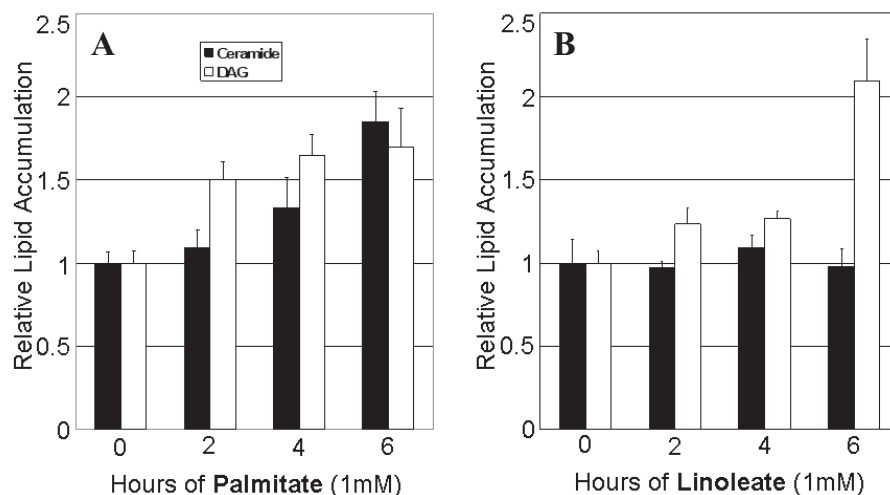
Sphingolipids are produced by a ubiquitous biosynthetic pathway initiated by the condensation of palmitoyl-CoA and serine, which is catalyzed by serine palmitoyltransferase (SPT) to produce 3-oxosphinganine. Three subsequent reactions follow, resulting in the production of sphinganine, dihydroceramide, and ultimately ceramide. Once generated, ceramide is the precursor of most active sphingolipids, including glucosylceramides, sphingosine, ceramide-1-phosphate, and sphingomyelin. SPT, which is rate-limiting for sphingolipid formation, is remarkably specific for palmitoyl-CoA.<sup>3</sup> Thus, the rate of sphingolipid biosynthesis is highly dependent upon

available long-chain fatty acids that can be metabolized to produce palmitate.<sup>4</sup> For example, maintaining isolated muscles in the presence of the saturated fatty acid palmitate is sufficient to drive formation of ceramide (Figure 3A). By contrast, incubating with the unsaturated fatty acid linoleate does not promote ceramide formation (Figure 3B). However, both lipids are sufficient to drive DAG synthesis.

Prior studies suggest that mitochondrial dysfunction may lead to impaired lipid oxidation, resulting in the aberrant accumulation of lipid metabolites that inhibit insulin action. For example, treating rats with the CPT1 inhibitor etomoxir promotes insulin resistance,<sup>5</sup> and overexpression of CPT1 in cultured muscle cells protects them from lipid-induced insulin resistance.<sup>6</sup> Moreover, overexpressing malonyl-CoA decarboxylase, which degrades malonyl-CoA and relieves its inhibition of CPT1, in the liver decreases FFA levels in serum and ameliorates insulin resistance caused by high fat feeding.<sup>7</sup> Lastly, some of the insulin-sensitizing effects of the drug metformin may result from its ability to promote lipid oxidation.<sup>8</sup>

## Glycerolipids

GPAT isozymes are located within the endoplasmic reticulum and the outer mitochondrial membrane, with the microsomal form(s) accounting for 90% of GPAT



**Figure 3.** Differential production of lipid metabolites from oversupply of palmitate or linoleate. Male Sprague-Dawley rats (150–200 g) were deeply anesthetized with sodium pentobarbital (110 mg/kg intraperitoneally), and soleus muscles were isolated, laterally bisected, and then transferred to 25-mL Erlenmeyer flasks containing 2 mL of Kreb's buffer supplemented with 2.5% bovine serum albumin, 8 mM glucose, and 1 mM HEPES (pH 7.2). Muscles were maintained for 6 hours in a shaking water bath at 29°C while being continuously gassed with 95% O<sub>2</sub>/5% CO<sub>2</sub>. Tissues were trimmed of tendons and rapidly frozen in liquid nitrogen. Lipids were extracted, phosphorylated with the diacylglycerol (DAG)-kinase, resolved by thin-layer chromatography, and detected using a Storm phosphorimager.

activity in most tissues.<sup>9</sup> An exception is the liver, where mitochondrial GPAT (mtGPAT) metabolizes a greater percentage of FFAs destined to become glycerolipids. While the endoplasmic reticulum forms have not been cloned, studies on the mitochondrial forms have shed insight into the role of this class of enzymes in the modulation of obesity and insulin sensitivity. Knockout mice lacking mtGPAT isoform 1 (mtGPAT1) have a smaller fat pad, lower body weight, a lower rate of VLDL secretion, and are protected from insulin resistance caused by high fat feeding.<sup>9,10</sup> The use of recombinant adenoviruses encoding shRNA constructs targeting degradation of the mtGPAT1 transcript produced similar findings, with animals demonstrating an improved metabolic profile characterized by decreased fasting glucose and triglyceride levels in the circulation.<sup>11</sup> When compared with wild-type mice, animals lacking mtGPAT1 demonstrated markedly lower levels of hepatic triacylglycerol and diacylglycerol, which have been implicated as potential modulators of insulin sensitivity.<sup>9</sup>

### Diacylglycerol

DAG has long been suspected to be a key lipid intermediate linking nutrient excess to the antagonism of insulin signaling,<sup>12</sup> and the molecule has been shown to accumulate in muscles obtained from insulin-resistant rodents and humans.<sup>13,14</sup> Much of the work implicating DAG in the induction of insulin resistance has been performed by the Shulman laboratory, which has found

that infusing a triglyceride emulsion enriched in the acyl chain linoleate (Liposyn II, Abbott, North Chicago, IL, 65.8% linoleate) induces insulin resistance and promotes the accumulation of DAG (predominantly containing one or more linoleate acyl chain). This group found that the infusion of this lipid cocktail enhances serine/threonine phosphorylation and inhibition of IRS-1, which antagonizes its ability to activate PI3K.<sup>5</sup> Knockout mice lacking one or more copies of PKC $\theta$ <sup>12</sup> or I $\kappa$ K $\beta$ <sup>10</sup> are protected from liposyn II-induced insulin resistance, suggesting their involvement in a signaling pathway linking intracellular lipid metabolites to the antagonism of insulin action. To tease out the relative order of events, Gao et al.<sup>15</sup> mapped the signaling pathway linking linoleate to the antagonism of IRS-1 in 3T3-L1 adipocytes. Briefly, they determined that linoleate activated PKC $\theta$ , which in turn activated I $\kappa$ K $\beta$  and JNK to promote phosphorylation of IRS-1 on Ser307. Interestingly, Li et al.<sup>16</sup> reported that PKC $\theta$  phosphorylates IRS-1 on Ser1101. The aforementioned studies evaluating the consequence of hepatic GPAT1 ablation are consistent with this hypothesis, as GPAT1 knockdown decreased membrane association (i.e., activation) of PKC $\theta$  in association with a reduction in DAG and improvement of glucose tolerance.<sup>11</sup> In addition to PKC $\theta$ , PKC $\delta$  and PKC $\epsilon$  are activated in tissues obtained from insulin-resistant rodents.

Despite these data supporting a role for DAG in insulin resistance, questions remain regarding whether DAG plays a quantitatively significant role in muscle

glucose uptake. First, phorbol esters that mimic DAG have often been shown to have no inhibitory effect in insulin action.<sup>17,18</sup> Second, DAG produced after incubating culture myotubes<sup>4</sup> or isolated muscles (unpublished observation) in palmitate is insufficient to inhibit insulin signaling (Figure 4). Third, overexpressing serine palmitoyltransferase, the rate-limiting enzyme in the pathway leading to sphingolipid synthesis, blocks palmitate induction of DAG (likely by directing fatty acyl-CoAs into sphingolipids), but not its perturbation of insulin signaling (Figure 3). One possible explanation for this discrepancy is that DAG comprised of 18:2 is a more potent activator of this pathway than that derived from saturated fats. Indeed, Wakelam<sup>19</sup> described numerous experimental findings supporting the hypothesis that polyunsaturated forms of DAG, and not saturated derivatives, serve as intracellular signals. An important step will be for researchers to determine whether selectively modulating intracellular DAG levels has a marked effect on insulin sensitivity *in vivo*.

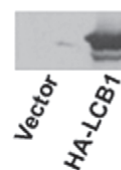
### Sphingolipids

Although sphingolipids are less abundant than glycerolipids, numerous studies suggest that they may play an important role in the regulation of insulin sensitivity during times of metabolic stress. First, inflammatory cytokines and glucocorticoids, which have been implicated as factors linking obesity to the antagonism of insulin action, selectively induce sphingolipid synthesis without affecting the glycerolipid pathway. Thus, rates of sphingolipid synthesis are influenced by excessive fat intake and adipokines implicated in insulin resistance. Second, certain sphingolipid metabolites have been shown to inhibit insulin signaling and action (e.g., ceramide and ganglioside GM3, discussed below). Third, in cultured cell models aimed at mimicking lipid-induction of insulin resistance, inhibitors of sphingolipid synthesis restored insulin sensitivity.<sup>20,21</sup>

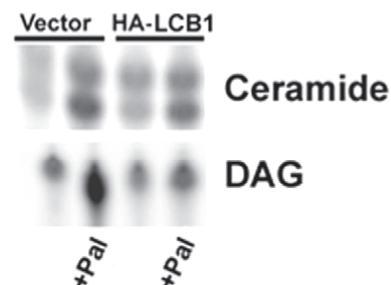
### Ceramide

Ceramide levels are elevated in muscles or liver from insulin-resistant rodents<sup>22,23</sup> or humans.<sup>24,25</sup> When added to cultured cells, ceramide analogs inhibit insulin-stimulated glucose uptake, GLUT4 translocation, and/or glycogen synthesis. In cultured muscle,<sup>20,26,27</sup> adipocytes,<sup>28</sup> and hepatocytes (Figure 5), ceramide analogs have been shown to inhibit activation of Akt/protein kinase B (PKB), which underlies its rapid effects on glucose uptake and anabolic metabolism. Ceramide appears to inhibit this signaling step through two independent mechanisms, depending on the tissue being studied. First, ceramide promotes the dephosphorylation of Akt/

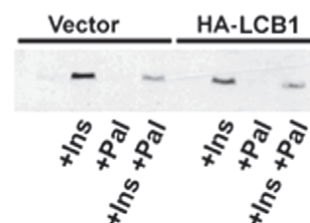
### A. Western Blot: Anti-HA



### B. DAG/Ceramide Assay



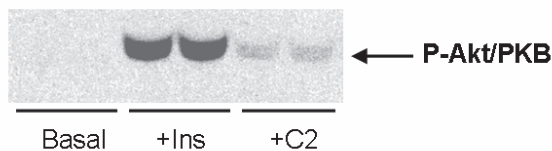
### C. Western Blot: Anti-PAkt



**Figure 4.** Palmitate inhibits Akt/protein kinase B phosphorylation in C2C12 myotubes overexpressing LCB1. A, Stable C2C12 myotubes expressing an empty vector or HA-LCB1 were lysed, resolved by SDS-PAGE, transferred to nitrocellulose, and immunoblotted with an anti-HA antibody. Data are representative of three independent experiments. B, The cell lines shown in A above were treated with or without palmitate (Pal, 0.75 mM) for 8 hours. Ceramide and diacylglycerol (DAG) were quantified as in Figure 3. Shown are the portions of the autoradiogram that co-migrated with ceramide or DAG standards. Data are representative of three independent experiments. C, The cell lines shown in A above were treated with or without palmitate (Pal, 0.75 mM) for 8 hours, with insulin present (Ins) or absent for the final 10 minutes. Cells were prepared as in A above and then immunoblotted with anti-phospho-Akt/PKB antibodies. Data are representative of three independent experiments.

PKB by directly activating protein phosphatase 2A (PP2A), which is the primary phosphatase responsible for dephosphorylating Akt/PKB.<sup>29</sup> Second, ceramide inhibits the translocation and activation of Akt/PKB, which





**Figure 5.** Ceramide inhibits Akt/protein kinase B phosphorylation in cultured hepatocytes. H4IIE cells were serum-deprived for 2 hours prior to treating with or without insulin (1  $\mu$ M, 10 min) or C2-ceramide (100  $\mu$ M, 20 min). Cells were lysed in SDS, and proteins were resolved by SDS-PAGE, transferred to PVDF membranes, and immunoblotted with antibodies recognizing the phosphorylated S473 phosphorylation site.

it appears to accomplish through the activation of PKC $\zeta$ , which phosphorylates Akt/PKB on an inhibitory residue present in the enzyme's pleckstrin homology domain.<sup>21,26</sup>

Ceramide can be rapidly deacylated (by ceramidases), phosphorylated (by ceramide kinase), or glucosylated (by glucosylceramide synthase) to produce a broad array of additional molecules with a sphingosine backbone. Both sphingosine and glucosylceramide (discussed below) have been shown to inhibit insulin stimulation of glucose transport. However, in our studies using cultured C2C12 myotubes or 3T3-L1 adipocytes, neither appeared to be the primary intermediate linking exogenous fats to the inhibition of insulin signaling. Specifically, inhibitors of glucosylceramide synthase and acid ceramidase mimicked and exacerbated lipid effects on ceramide accumulation while inhibiting insulin signaling to Akt/PKB.<sup>20</sup> Moreover, overexpression of acid ceramidase negated the antagonistic effects of exogenous palmitate.<sup>30</sup> Thus, our studies were consistent with ceramide, rather than a glucosylated or deacylated ceramide metabolite, being the primary intermediate linking exogenous fats to the inhibition of insulin signaling.

Despite these data identifying ceramide as a likely intermediate accounting for some of palmitate's antagonistic effects, some researchers have failed to observe changes in ceramide in rodent models of insulin resistance. For example, when infused into rodents, Liposyn II antagonizes glucose homeostasis but fails to promote intramuscular ceramide accumulation.<sup>13,31</sup> Given the unsaturated nature of the fatty acids in this cocktail, its failure to induce ceramide may have been expected, as sphingolipids require palmitate for the creation of the sphingosine moiety<sup>3</sup> (Figure 3). Nonetheless, these studies clearly indicate that lipids can induce insulin resistance in muscle via a ceramide-independent mechanism. Thus, an important question yet to be addressed is whether blocking ceramide accrual has a quantitatively significant impact on insulin sensitivity and glucose homeostasis in vivo.

## Glucosylceramides

The ganglioside GM3 has also been identified as a potential antagonist of insulin signaling, apparently by inhibiting the dimerization and tyrosine phosphorylation of several tyrosine kinase receptors.<sup>32</sup> Adding exogenous GM3 to adipocytes has been shown to suppress insulin-stimulated tyrosine phosphorylation of its receptor and IRS-1 while inhibiting glucose uptake. Kabayami et al.<sup>33</sup> presented evidence that this effect is due to GM3's ability to displace insulin receptors from detergent-resistant raft domains.

GM3 synthase transcript levels are elevated in white adipose tissue of obese rodents,<sup>34</sup> and the implementation of pharmacological and genetic ablation strategies to inhibit glucosylceramide or GM3 synthase activity improves insulin sensitivity and glucose homeostasis.<sup>35,36</sup> For example, the use of a glucosylceramide synthase inhibitor prevented TNF- $\alpha$ -induced defects in insulin signaling.<sup>35</sup> Moreover, mice lacking GM3 synthase (CMP-NeuAc:lactosylceramide  $\alpha$ 2,3-sialyltransferase) demonstrated increased sensitivity to insulin, enhanced insulin receptor phosphorylation in skeletal muscle, and protection from high fat diet-induced insulin resistance.<sup>36</sup> Collectively, these data suggest that therapeutic interventions aimed at reducing GM3 levels in vivo could be a useful treatment strategy for combating insulin resistance.

## Cholesterol

In some studies, inhibitors of cholesterol synthesis (i.e., statins) have been shown to improve insulin sensitivity in insulin-resistant rodents and humans.<sup>37,38</sup> Many investigators have hypothesized that the improvement of insulin sensitivity derives from the associated decrease in VLDL production, and thus could reduce the delivery of triglycerides to peripheral tissues. In addition, however, cell-autonomous effects of cholesterol depletion have been observed. For example, in cultured cells, cholesterol reduction is associated with an enhancement of insulin's metabolic signaling, while not affecting pathways involved in mitogenesis.<sup>39</sup> Moreover, in the absence of observed effects on insulin signaling to Akt, a reduction in plasma membrane cholesterol was shown to be associated with an increase in basal and insulin-stimulated translocation of GLUT4, the glucose transporter isoform that mediates insulin-stimulated hexose uptake.<sup>40-42</sup>

## EXTRACELLULAR LIPIDS AS REGULATORS OF INSULIN ACTION

Recent studies reveal that non-esterified fatty acids may also induce insulin resistance by activating toll-like

receptors 2 or 4, which are present on adipose, muscle, and/or liver cells.<sup>43</sup> Fatty acids inhibit insulin signaling, activate JNK and I $\kappa$ K $\beta$ , and induce inflammatory cytokines (e.g., interleukin-6 or TNF- $\alpha$ ). The inclusion of inhibitory antibodies blocking the activation of TLR-2 or siRNA targeting degradation of the TLR2 or its adaptor protein, MyD88 negates these effects.<sup>44,45</sup> Moreover, mice lacking TLR4 are protected from insulin resistance caused by lipid infusion or high-fat diets, and exogenous FFAs fail to induce cytokines in adipocytes obtained from these knockout animals.<sup>46</sup> Interestingly, TLR-2 shows a significantly higher frequency of polymorphisms in populations at risk for metabolic disease.<sup>47</sup> Collectively, these studies provide a molecular explanation through which lipid oversupply triggers the inflammatory response associated with obesity.

## FUTURE CHALLENGES

We have described a number of lipid intermediates that appear capable of inhibiting insulin stimulation of glucose uptake or storage. Thus, biological systems have effectively developed a means of gauging available fuel sources as a means of altering relative rates of nutrient uptake, usage, or storage. However, while a clearer picture is developing with regard to which lipid metabolites selectively alter insulin signaling and action, questions persist about the quantitative importance of any of these individual lipid metabolites in vivo, as well as the mechanisms underlying these effects.

## ACKNOWLEDGMENTS

This work was supported by the National Institutes of Health (DK58784 and DK073181 to SAS and DK070565 to WLH), the American Heart Association (predoctoral/postdoctoral stipends to KLH and TAK, respectively), and the American Diabetes Association (research award to SAS).

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