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Integrating Mechanisms for Insulin Resistance: Common Threads and Missing Links

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Abstract

Insulin resistance is a complex metabolic disorder that defies a single etiological pathway. Accumulation of ectopic lipid metabolites, activation of the unfolded protein response (UPR) pathway and innate immune pathways have all been implicated in the pathogenesis of insulin resistance. However, these pathways are also closely linked to changes in fatty acid uptake, lipogenesis, and energy expenditure that can impact ectopic lipid deposition. Ultimately, accumulation of specific lipid metabolites (diacylglycerols and/or ceramides) in liver and skeletal muscle, may be a common pathway leading to impaired insulin signaling and insulin resistance.

All metazoa are heterotrophic; they need to eat. This defining characteristic poses a central challenge. Nutrient sources are often scarce and caloric demands constantly change. Animals solved this problem by developing integrated mechanisms to promote anabolism when calorie supply exceeds demands but readily become catabolic when demands cannot be met with consumption. The secretion and action of insulin (and related molecules in lower phyla) provided a solution to this central problem. Following nutrient consumption, insulin promotes carbohydrate uptake at key storage sites and prompts the conversion of carbohydrate and protein to lipids, a more efficient storage for calories.

Though this ability to store dietary energy for later times has supported the development of animal life for nearly 600 billion years, it has recently gone awry for humans. In a remarkably short time, we have altered an environment of caloric scarcity and high caloric demands into one with abundant caloric supply with very little caloric demands. Obesity is now endemic and societies are grappling with the rising prevalence of obesity-associated diseases, including the metabolic syndrome, nonalcoholic fatty liver disease (NAFLD), type 2 diabetes (T2D) and atherosclerotic heart disease. These diseases exact tremendous tolls on society, through both the loss of health and quality of life but also on health system resources. Insulin resistance is *sine quo non* with the pathogenesis for many of these modern diseases. Thus, understanding the pathogenesis of insulin resistance has become increasingly

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important to guide the development of future therapies and inform health and economic policy.

As stated above, insulin action essentially provides an integrated set of signals that allow us to balance nutrient availability and demands (Figure 1). There are diseases with impairments in insulin production, as in type 1 diabetes or in the monogenic maturity onset diabetes of the young (MODY) syndromes. These diseases are significant and can abruptly interrupt health, especially for children. In comparison, insulin resistance is insidious and affects a far greater number of people. By some estimates, within forty years, one in every three Americans will have type 2 diabetes (Boyle et al., 2010).

Here we review several mechanisms proposed to explain the pathogenesis of insulin resistance, mainly the development of insulin resistance from ectopic lipid accumulation, the development of “endoplasmic reticulum stress” and activation of the unfolded protein response and the contribution of systemic inflammation. Though many additional mechanisms have been offered, these three represent different aspects of metabolic control that ultimately may converge on common pathways to regulate insulin action.

1. Ectopic Lipid Accumulation

The association between lipids and insulin resistance is widely accepted. Early studies by Randle and colleagues in rodent heart and diaphragm muscle suggested that fatty acids impaired insulin-mediated glucose uptake in muscle by inhibition of pyruvate dehydrogenase leading to reductions in glucose oxidation (Randle et al., 1963). In rats, acute (<2 hours) lipid infusions decreased myocellular glucose utilization with the expected increases in intramyocellular glucose 6-phosphate (G-6-P) concentrations, as predicted by Randle’s hypothesis (Jucker et al., 1997). But, while this may hold true for acute experimental challenges, it does not explain insulin resistance in chronic disease states which can be attributed to reductions in both insulin-stimulated muscle glycogen synthesis and glucose oxidation (Shulman et al., 1990). Subsequent *in vivo* measurements of intramyocellular glucose and G-6-P concentrations in insulin resistant, type 2 diabetic subjects as well as during longer (3–6 hours) infusion of lipid in both rodents and humans have identified reductions in insulin-stimulated glucose transport activity, due to decreased insulin signaling, as the major factor responsible for causing insulin resistance in skeletal muscle (Cline et al., 1999; Dresner et al., 1999; Griffin et al., 1999; Roden et al., 1996). In parallel, molecular studies suggested that insulin resistance could be attributed to impaired GLUT4 translocation, largely due to defects in insulin signaling (Ciaraldi et al., 1995; Garvey et al., 1998) (Figure 1).

Lipids are clearly associated with insulin resistance. But, is it the circulating plasma lipids or the lipids accumulating within insulin responsive tissues that cause insulin resistance? Studies in normal weight, non-diabetic adults found that intramyocellular triglyceride content was a far stronger predictor of muscle insulin resistance than circulating fatty acids (Krssak et al., 1999), suggesting that intramyocellular lipids may cause muscle insulin resistance. This empiric observation was tested experimentally. In normal rats, Intralipid/heparin infusions, to raise plasma fatty acids, led to muscle insulin resistance concordant with the accumulation of intramyocellular diacylglycerol (DAG), and impairment in insulin signaling and muscle glucose uptake independent of changes in muscle triglyceride content thus dissociating muscle triglyceride concentrations from insulin resistance (Dresner et al., 1999; Yu et al., 2002). Diacylglycerols are signaling intermediates that activate members of the protein kinase C (PKC) family. In these experiments, muscle lipid accumulation was associated with activation of the novel PKC (nPKC) isoform PKCθ, providing a potential link between lipid accumulation and alteration in intracellular signaling. This link between

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DAG-mediated activation of PKC and muscle insulin resistance was replicated in human studies (Itani et al., 2002; Szendroedi et al., 2011). Though both of these studies have associated muscle diacylglycerol accumulation with nPKC activation, there are some differences. Lipids infused together with insulin over 6h result in activation of PKCδ (Itani et al., 2002). In contrast, PKCθ activation predominates when lipids were infused for 4h prior to insulin infusion (Szendroedi et al., 2011). Together, these studies support the paradigm that diacylglycerol accumulation in muscle can lead to muscle insulin resistance through activation of nPKCs (Figure 2).

Ectopic lipid accumulation also occurs in the liver. Nonalcoholic fatty liver disease (NAFLD) is now considered the most common chronic liver disease in the United States; by some estimates 50% of Americans will have NAFLD by 2030 (Younossi et al., 2011). Several human studies demonstrate the strong association between NAFLD *per se* and hepatic insulin resistance. Extreme examples are seen in individuals with severe congenital lipodystrophy. Affected individuals have little to no adipose tissue and store lipid ectopically. Consequently, they develop marked hepatic steatosis and hepatic insulin resistance. In these individuals, recombinant leptin therapy reduces caloric intake, and resolves NAFLD and hepatic insulin resistance (Petersen et al., 2002). Caloric restriction can also rapidly reduce hepatic steatosis in obese patients (Lim et al., 2011; Petersen et al., 2005). Subjects with poorly controlled T2D who were placed on a hypocaloric diet for up to 12 weeks experienced a marked and rapid decrease in liver fat content (~85%), associated specifically with a normalization in hepatic insulin sensitivity and reductions in fasting hyperglycemia and hepatic glucose production without changes in intramyocellular lipid or insulin mediated whole body glucose disposal (Petersen et al., 2005). Many individuals with NAFLD also have increased visceral adiposity, a marked expansion of the omental fat. While visceral adipose tissue has been implicated as causing hepatic insulin resistance, several studies present compelling data to the contrary. Patients with severe lipodystrophy (Petersen et al., 2002) and a mouse model of lipoatrophy (Kim et al., 2000) have no visceral fat but manifest severe hepatic resistance associated with severe hepatic steatosis. When the hepatic steatosis is reversed with leptin therapy in humans (Petersen et al., 2002) or fat transplantation in mice (Kim et al., 2000), hepatic insulin resistance resolves. Consistent with these observations Fabbrini *et al.* demonstrated hepatic insulin resistance was primarily related to intrahepatic lipid content, not visceral fat mass (Fabbrini et al., 2009). And, they found that surgical removal of visceral adipose tissue did not alter glucose homeostasis or insulin sensitivity (Fabbrini et al., 2010)

Genetic rodent models, which alter expression of lipid transport proteins, provide evidence for the role of ectopic lipid accumulation in the pathogenesis of insulin resistance. Lipoprotein lipase (LpL) is a key enzyme that hydrolyzes circulating triglyceride permitting tissue uptake through specific fatty acid transport proteins (FATPs) together with CD36. Muscle specific overexpression of lipoprotein lipase (LpL) promotes muscle lipid uptake and muscle insulin resistance (Kim et al., 2001). In contrast, deletion of LpL (Wang et al., 2009), or other proteins involved in fat transport, such as CD36 (Goudriaan et al., 2003; Hajri et al., 2002) or FATP1 (Kim et al., 2004b) protects mice from muscle lipid accumulation and muscle insulin resistance when challenged with high-fat diets (Figure 2). Similarly, hepatic specific overexpression of LpL leads specifically to hepatic steatosis and hepatic insulin resistance (Kim et al., 2001; Merkel et al., 1998). Adenoviral mediated overexpression of hepatic CD36 caused NAFLD, even in regular chow-fed mice (Koonen et al., 2007). Loss of hepatic fatty acid transport, specifically through deletion of FATP2 (Falcon et al., 2010) or FATP5 (Doege et al., 2008) protects against the development of hepatic steatosis and glucose intolerance (Figure 3).

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The liver also actively exports lipids. The importance of this balance between hepatic lipid uptake and export is demonstrated in the mice that overexpress human apolipoprotein CIII (ApoC3). This lipoprotein can inhibit LpL activity and limit peripheral fat uptake thereby promoting postprandial hyperlipidemia. While transgenic mice that overexpress ApoC3 (ApoC3 tg) have marked hypertriglyceridemia, hepatic lipid content in regular chow fed mice is not different than wild type mice fed the same diet (Lee et al., 2011b). However, when placed on a high-fat diet, ApoC3 tg mice develop hepatic steatosis with diacylglycerol accumulation, PKCε activation and hepatic insulin resistance (Lee et al., 2011b). The development of hepatic steatosis could be attributed to a mismatch between hepatic lipid uptake and lipid export in the high fat fed mice; hepatic lipid uptake was increased under both conditions, but export, via ApoB100 containing VLDL particles, was decreased in the hyperinsulinemic fat-fed mice due to suppression of ApoB100 expression. This has relevance to human disease. Lean individuals (body mass index less than 25 kg/m^2) who carry polymorphisms in the insulin response element of the ApoC3 gene (rs2854116 and rs2854117) have increased fasting plasma ApoC3 concentrations and fasting hypertriglyceridemia (Petersen et al., 2010). This was associated with a decrease in plasma lipid clearance after both an oral and intravenous lipid challenge and an increase in hepatic steatosis. Thus, as with the ApoC3 tg mice, when individuals carrying polymorphisms in ApoC3 exist in “toxic environments” (Novak and Brownell, 2011), they are prone to NAFLD and hepatic insulin resistance. Importantly, this subtle, gene-environment relationship is not evident in obese subjects who already have a high prevalence of NAFLD (Kozlitina et al., 2010). Thus, ectopic lipid accumulation in liver either from increased delivery or decreased export, can lead to hepatic insulin resistance. While genetic defects in hepatic mitochondrial fatty acid oxidation (e.g. mice lacking long-chain acyl-CoA dehydrogenase, LCAD) can also be a predisposing condition to hepatic steatosis and hepatic insulin resistance (Zhang et al., 2007) studies in patients with NAFLD are few and differ in their conclusions with some studies reporting reduced (Cortez-Pinto et al., 1999; Schmid et al., 2011) while others report increased hepatic mitochondrial metabolism (Sunny et al., 2011).

Lipid Mediators of Insulin Resistance

Lipids, as signaling intermediates, encompass a vast range of molecules with distinct functions. While the earliest recognized lipid signals were circulating lipids, endotoxins and prostaglandins, intracellular lipid intermediates (e.g. diacylglycerols, ceramides, PIP3, etc.) also mediate intracellular signaling. The local production of these lipid metabolites has been thought to provide some degree of signaling localization. That is, signaling events and therefore cellular actions can be directed to specific regions within the cell in response to the generation of a key lipid intermediate. So, what is the pathological lipid moiety? Upon cellular entry, fatty acids are rapidly esterified with coenzyme A to for fatty acyl-CoA’s. These are successively transferred to a glycerol backbone to form mono-, di- and tri-acylglycerols. They can also esterify with sphingosine to form ceramides. Some of these lipid intermediates (e.g. diacylglycerol and ceramides) are also known to function as second messengers in key signaling pathways. These lipid intermediates comprise a lineup of metabolic suspects implicated in the pathogenesis insulin resistance. These associations can be directly tested using genetic mouse models that modulate the expression of the enzymes involved in lipid metabolism.

Mitochondrial acyl-CoA:glycerol-sn-3-phosphate acyltransferase (mtGPAT) catalyzes the formation of lysophosphatidic acid from fatty acyl CoA and glycerol 3-phosphate (Figure 3). When mtGPAT deficient ($mtGPAT1^{-/-}$) mice are placed on a high-fat diet, they accumulate hepatic fatty acyl-CoA but not hepatic diacylglycerol and triglyceride (Neschen et al., 2005). Despite the twofold increase in fatty acyl-CoA, $mtGPAT1^{-/-}$ mice are protected from diet-

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induced hepatic insulin resistance. Similarly, overexpression of mtGPAT in rats increases hepatic DAG and leads to hepatic insulin resistance (Nagle et al., 2007). Together, these studies suggest that fatty acyl CoA's do not cause insulin resistance.

Several lines of evidence support a role for ceramides in the pathogenesis of insulin resistance. Ceramides are primarily membrane lipids, a precursor in the formation of sphingomyelin. However, increases in hepatic and muscle ceramide content, along with diacylglycerols, were associated with insulin resistance in obese Zucker (*fa/fa* rats, homozygous for a truncated, nonfunctional leptin receptor) (Turinsky et al., 1990). Treating fat-fed mice with myriocin, an inhibitor of serine palmitoyl transferase 1, specifically attenuates the increase in muscle ceramides in fat-fed mice without any change in long chain acyl-CoA's, diacylglycerol or triglyceride, and improves glucose tolerance. The role of ceramides as a mediator of insulin resistance may be limited to saturated fats. Myriocin prevents acute skeletal muscle insulin resistance following infusion of palmitate, but not oleate (Holland et al., 2007). Mice fed 12 weeks of a lard-based diet (60% fat calories) were protected from glucose intolerance when treated with myriocin (Ussher et al., 2010). Ceramide synthesis in response to palmitate may occur upon activation of inflammatory pathways. Palmitate infusion increases plasma cytokine concentrations and mice lacking toll-like receptor 4 (TLR4) are protected from ceramide accumulation and insulin resistance following lard, but not soy oil infusions (Holland et al., 2011). Some have also suggested that saturated fatty acids may also be the ligands for TLR4 (Shi et al., 2006). Thus, intracellular ceramides may also act as "second messengers" that coordinate a cells response to circulating cytokines or possibly nutrient (e.g. saturated fatty acids) signals.

In some instances, diacylglycerols also function as second messengers (specifically sn1,2 diacylglycerol), canonically produced from phosphatidylinositol 4,5-bisphosphate by the action of phospholipase C. In contrast, with ectopic lipid accumulation in insulin resistant states, intracellular diacylglycerol accumulation can be secondary to a mismatch in the rate of diacylglycerol synthesis exceeding the rate of its incorporation into triacylglyerol, or triglyceride hydrolysis pathways. Thus, diacylglycerols are metabolites that also have intracellular signaling properties. This association between diacylglycerol and insulin resistance can be illustrated in mice with genetic manipulations of the Acyl-CoA:diacylglycerol acyltransferase (DGAT), the enzymes that acylate diacylglycerols into triglycerides. Mice that overexpress skeletal muscle DGAT1 (MCK-DGAT1) accumulate muscle triglyceride but are protected from lipid-induced muscle insulin resistance (Liu et al., 2007) and recapitulate the athlete's paradox, in which endurance athletes have increased muscle triglyceride yet are insulin sensitive (Goodpaster et al., 2001; Krssak et al., 2000). Though muscle triglyceride content was increased in the MCK-DGAT1, muscle DAG content was lower and may account for the protection from lipid-induced insulin resistance. DAG's can also be converted into phosphatidic acid, a major membrane lipid, by diacylglycerol kinases (DGK's) (Chibalin et al., 2008). DGKδ expression was found decreased in skeletal muscles of both hyperglycemic rodents and poorly controlled diabetic patients. In mice, DGKδ haploinsufficiency ($DGK\delta^{+/-}$) increased muscle DAG, but not triglyceride, and resulted specifically in muscle, insulin resistance. Taken together with the previous studies on GPAT, these studies exonerate tissue fatty acyl CoA and triglyceride as pathogenic lipid species for insulin resistance.

Other genetic studies of the DGAT enzyme family reveal a complicated relationship between DGAT, its substrates and its product. Considering the results in the MCK-DGAT1 mice, deletion of DGAT1 should specifically promote DAG, but not triglyceride, accumulation and lead to insulin resistance. In actuality, DGAT1 knockout mice have reductions in *both* tissue triglyceride and diacylglycerol content (Smith et al., 2000), are resistant to diet-induced obesity and insulin resistance. DGAT2, which is normally highly

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expressed in white adipose and liver, is thought to be a more potent diacylglycerol acyltransferase. Transgenic overexpression of DGAT2 in glycolytic fibers (MCK-DGAT2) promotes muscle triglyceride accumulation and decreases diacylglycerol content, as expected, but surprisingly increases muscle fatty acyl CoA's and ceramide content (Levin et al., 2007). MCK-DGAT2 mice develop modest muscle insulin resistance and suggest that other lipid metabolites (e.g. ceramides) may also mediate the development of insulin resistance. In contrast, transgenic overexpression of hepatic DGAT2 increases hepatic TAG and DAG content ~2.5–3 fold and hepatic ceramide content by ~10–15% and is associated with hepatic insulin resistance (Jornayvaz et al., 2011). Decreasing hepatic and adipose DGAT2 expression with specific antisense oligonucleotides (ASO's) lowers diacylglycerol content by inhibiting lipogenesis, and reverses diet-induced hepatic insulin resistance (Choi et al., 2007). These studies demonstrate a sometimes paradoxical effect of DGAT modulation on hepatic diacylglycerol content. Overexpression can at times increase lipid intermediates that are proximal to DGAT (as in muscle and liver DGAT2 transgenics) and inhibition can decrease proximal metabolites. In the latter case, it is possible that transient increases in early lipid intermediates (e.g. fatty acyl CoA's) may serve to suppress the lipogenic pathway (Choi et al., 2007). Though the underlying cellular mechanisms accounting for lipid changes may be counterintuitive, the results from these studies do support the association between insulin resistance and the accumulation of both diacylglycerols and ceramides.

Intracellular lipid localization and insulin resistance

Most studies relating ectopic lipid accumulation to insulin resistance focus on the total lipid content within key tissues. But, does it matter where within the cell the specific lipids are localized? Lipids are, after all, pervasive cell constituents, present in all membranes, in lipid droplets and esterified to various carriers (e.g. coenzyme A and carnitine). So, does the lipid content in different intracellular compartments affect the development of insulin resistance? A definitive answer is not available, but some recent studies suggest that subcellular localization does matter with regards to insulin resistance.

Intracellular lipid droplets or "adiposomes" (Liu et al., 2004) are now considered sites of active lipid synthesis and lipolysis (Figures 2 and 3). They possess a mantle of enzymes that regulate the entry and exit of lipid species. In rare instances, mutations in some lipid droplet proteins are associated with congenital lipodystrophy syndromes. But, recently genome wide array screens have associated NAFLD with a relatively common single nucleotide polymorphism, rs738409, in the lipid droplet protein patatin-like phospholipase domain-containing protein 3 (PNLPA3) (Romeo et al., 2008). This protein, also called adiponutrin, encodes a protein that possesses both triglyceride lipase and transacylation activity (Jenkins et al., 2004). The rs738409 polymorphism is a I148M substitution in the protein, that results in a loss of lipolytic activity. Though carriers of this SNP have increased liver fat content, most reports suggest it is not associated with insulin resistance (Kantartzis et al., 2009; Kotronen et al., 2009; Speliotes et al., 2010). A recent study demonstrated that pediatric carriers have higher liver triglyceride but without any change in hepatic insulin sensitivity relative to control subjects (Santoro et al., 2010). Though these data pose a compelling challenge to the notion that ectopic lipid accumulation causes insulin resistance, the subjects in many of these studies were already obese and even the control subjects were relatively insulin resistant. Thus, to truly discern whether PNPLA3 confers a risk of steatosis without additional insulin resistance, hepatic insulin sensitivity will need to be assessed between lean PNPLA3 wild-type individuals with NAFLD and lean PNPLA3 variant carriers with NAFLD.

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Rodent studies exploring the function of PNPLA3 have so far not helped to resolve this quandary. Adenoviral overexpression of the mutant gene in mice increases lipid droplet size and triglyceride accumulation *in vivo*, consistent with the human data (He et al., 2010). And there is consensus that PNPLA3 expression is regulated by SREBP1c and perhaps by ChREBP (Dubuquoy et al., 2011; Huang et al., 2010; Qiao et al., 2011). However, the exact role of PNPLA3 in regulating lipid metabolism remains unknown. *A priori*, if PNPLA3 functions as a lipase, increased hepatic PNPLA3 expression should promote lipolysis and decrease liver lipid content. However, overexpression of the wild-type PNPLA3 does not alter liver lipid content, (He et al., 2010; Qiao et al., 2011). In addition, PNPLA3 knockout mice do not develop hepatic steatosis and do not appear to have altered glucose homeostasis, even when provoked with a variety of dietary challenges (Basantani et al., 2011; Chen et al., 2010). This putative lipase also possesses transacylation activity and thus may also have a role in lipid synthesis (Jenkins et al., 2004). PNPLA3 expression in adipose tissue (Caimari et al., 2007; Kershaw et al., 2006) and liver (Huang et al., 2010) decreases with fasting and increases in response to insulin and glucose; opposite of what would be expected from a triglyceride lipase and more consistent with a potential role in lipogenesis. Additional studies are needed to better understand the role of PNPLA3 in regulating lipid homeostasis, specifically how polymorphisms may affect its transacylase and lipolytic function and whether this alters the accumulation of key signaling lipid metabolites, e.g. diacylglycerol or ceramides.

In contrast, adipose triglyceride lipase (ATGL, also known as desnutrin and PNPLA2) is a potent lipase that catalyzes the hydrolysis of triglycerides into diacylglycerols. Mice lacking ATGL have marked alterations in lipid metabolism with ectopic lipid accumulation in most tissues, manifesting dramatically with massive cardiac lipid accumulation leading to premature death (Haemmerle et al., 2006). Despite adiposity and ectopic lipid accumulation, glucose tolerance was improved, again exculpating triglycerides in the pathogenesis of insulin resistance. Recent studies in humans have found that ATGL protein expression in skeletal muscle is inversely related to whole body insulin-stimulated glucose disposal in humans (Badin et al., 2011). A possible mechanistic link is proposed from *in vitro* experiments. Overexpression of ATGL in cultured myotubules increases both diacylglycerol and ceramide content and is associated with impaired insulin signaling. The non-selective PKC inhibitor calphostin-C prevents this impairment in insulin signaling, suggesting that PKC activation is required for ATGL mediated insulin resistance (Badin et al., 2011).

ATLG exerts profound influence over whole body energy metabolism. $ATGL^{-/-}$ mice have impaired thermogenesis when exposed to cold (Haemmerle et al., 2006). These mice are more reliant on carbohydrate oxidation during the light phase (fasting) of the diurnal cycle and when subjected to exercise, have a greater depletion of muscle and liver glycogen, consistent with an inability to switch to a lipid fuel source (Huijsman et al., 2009). Additional insights are gleaned from tissue specific modulation of ATGL expression. Adipose specific deletion of ATGL recapitulates some of the phenotype of the whole body knockout, with decreased energy expenditure and thermogenesis. Yet, this still prevents diet-induced insulin resistance attributable to a decrease in liver diacylglycerol content (Ahmadian et al., 2011). Surprisingly, adipose specific overexpression of ATGL also appears to protect mice from insulin resistance. This phenotype is thought due to an increase in futile lipid cycling and increased white adipose tissue UCP1 expression, which increases energy expenditure (Ahmadian et al., 2009). Liver specific deletion of ATGL render mice to be more prone to hepatic steatosis and decreased lipid oxidation, but without alteration in glucose tolerance (Wu et al., 2011). In comparison, adenoviral overexpression of hepatic ATGL reduced liver lipids (including DAGs and ceramides) possibly due to decreased lipogenesis and increased lipid oxidation and was associated with increased insulin sensitivity during an insulin tolerance test (Turpin et al., 2011). Together these studies

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suggest a role for ATGL both in regulating energy balance and the generation of key lipid intermediates. Without ATGL, cells cannot readily access the energy stored in triglycerides, but also cannot generate the lipid mediators of insulin resistance. And, forced overexpression may trigger an imbalance between synthesis and storage, establishing futile cycles that waste energy.

ATGL is a tightly regulated enzyme, through both modification [e.g. phosphorylation by AMPK (Ahmadian et al., 2011)] and regulatory proteins. ATGL is activated by comparative gene identification-58 (CGI-58) protein and pigment epithelial derived factor (PEDF) and inhibited by the G_0/G_1 switch gene 2 (G0S2). Antisense oligonucleotide mediated knockdown of the comparative gene identification-58 (CGI-58) protein, a key activator of ATGL, markedly increased hepatic TAG, DAG and ceramide in fat-fed mice (Brown et al., 2010). Despite the increases in these lipid species, knockdown of CGI-58 was associated with improved glucose tolerance and insulin sensitivity. These studies also challenge the association between ectopic lipid accumulation and insulin resistance and suggest that additional levels of control are present. ATGL can also be activated by the pigment epithelial derived factor (PEDF) (Chung et al., 2008). Five days of exogenous PEDF administration markedly decreased muscle triglyceride content, though not diacylglycerol or ceramide content (Borg et al., 2011). Insulin tolerance tests performed under these conditions revealed a more rapid recovery of blood glucose after insulin in wild-type mice treated with PEDF but not $ATGL^{-/-}$ mice, suggesting that the effects of PEDF are ATGL dependent. In contrast to CGI-58 and PEDF, G0S2 inhibits ATGL activity (Yang et al., 2010b). Though expression of G0S2 can inhibit lipolysis, there are no data on how it may modulate insulin sensitivity, specifically whether it could improve insulin sensitivity as in genetic deletions of ATGL.

Recently, studies in mice that overexpressed DGAT2 have suggested that the intracellular localization of diacylglycerol is important (Jornayvaz et al., 2011). This enzyme is localized to the endoplasmic reticulum but, with lipid accumulation, is highly expressed in the lipid droplet membranes (Stone et al., 2009). Mice overexpressing hepatic DGAT2 develop hepatic steatosis (Monetti et al., 2007), associated with hepatic but not peripheral insulin resistance (Jornayvaz et al., 2011). Whereas total DAG content was only modestly increased in the DGAT2 transgenic mice (specifically the “low-overexpressing” strain), the DAG content in the cytosolic fraction containing the lipid droplets was increased nearly 10-fold and associated with activation of PKCε, impairment of insulin signaling and hepatic insulin resistance. Though preliminary, these data, together with our growing understanding of the biology of lipid droplets suggest that trafficking of lipids from this particular cellular organelle may play a key role in the development of insulin resistance.

Connecting the chain of events: From lipid accumulation to insulin resistance

Diacylglycerol and novel Protein Kinase Cs

Protein kinase C family members are ideal for sensing a lipid signal and, through kinase activity, affecting cellular events in response. The PKC kinases belong to a larger family of AGC kinase that include a broad array of serine/threonine kinases and are themselves further subdivided into three categories: conventional PKC’s (cPKC: α, βI, βII and γ) that require both calcium and diacylglycerol for activation, novel PKC’s (nPKC: δ, ε, η, and θ) that require only diacylglycerol and atypical PKC’s (aPKC: ζ and λ) that require neither calcium nor diacylglycerol. Of interest, sequence analyses suggest that the atypical PKC’s may in fact be the most ancient, suggesting that as biological systems became more complex, PKC’s evolved into additional signaling roles (Miyake et al., 2009).

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Earlier studies demonstrated that activation of PKC's with phorbol acetate caused insulin resistance (Haring et al., 1986; Jacobs et al., 1983; Karasik et al., 1990) and that PKC's were activated in diabetic rodent models (Avignon et al., 1996; Considine et al., 1995). In rodents, an infusion of Intralipid/heparin to raise plasma fatty acid concentrations leads to PKC-θ activation, which is associated with muscle insulin resistance and reductions in insulin-stimulated IRS-1 tyrosine phosphorylation and IRS-1 associated PI 3-kinase activity (Griffin et al., 1999) associated with an increase in intracellular diacylglycerol content but independent of changes in intramuscular ceramide and triglyceride content (Yu et al., 2002). Mice lacking PKCθ are protected from skeletal muscle insulin resistance under a similar acute lipid infusion (Kim et al., 2004a). In contrast, following 14-weeks of high-fat feeding, PKCθ knockout mouse are prone to diet-induced obesity and, in this setting, develop muscle, adipose and hepatic insulin resistance (Gao et al., 2007). PKCθ knockout mice have decreased locomotor activity and energy expenditure. Thus, while lack of PKCθ may protect from acute lipid-induced muscle insulin resistance, chronic high-fat diet can still lead to insulin resistance, which can likely be attributed to the development of hepatic insulin resistance, which would not be prevented by deletion of PKCθ. In addition it is possible that under chronic high-fat conditions other nPKC isoforms may compensate for PKC-θ deficiency and eventually allow the development of insulin resistance in skeletal muscle.

PKC's also play a role in the development of hepatic insulin resistance. In rodents, three days of high-fat feeding causes marked hepatic steatosis without peripheral adiposity or increase in muscle lipid content. With this intervention, hepatic insulin resistance develops without muscle or adipose insulin resistance. In this setting, hepatic insulin resistance is associated specifically with activation of PKCε. Decreasing PKCε expression using a specific ASO enhanced hepatic insulin response in fat-fed rats despite hepatic steatosis, suggesting that PKCε is required for the development of hepatic insulin resistance in NAFLD. Consistent with these findings, PKCε knockout mice (*Prkce*$^{-/-}$) are protected from diet-induced insulin resistance following one week of high-fat feeding (Raddatz et al., 2011) despite subtle increases in liver lipid content. Interestingly, *Prkce*$^{-/-}$ were prone to hepatic steatosis with chronic (16 weeks) high-fat feeding. Despite this, *Prkce*$^{-/-}$mice continued to display enhanced glucose tolerance, though at this time point, this finding was attributed to increased insulin secretion.

PKCδ, a novel isoform like PKCε, has also been implicated in the pathogenesis of hepatic insulin resistance. PKCδ is activated in the livers of rats subjected to a six hour Intralipid/heparin infusion, concomitant with the development of hepatic insulin resistance (Lam et al., 2002). However, the exact role of PKCδ in the development of hepatic insulin resistance remains unclear. During a lipid infusion, PKCδ activation occurs simultaneously with activation of IKK-β, raising the possibility that PKCδ may also be responding to a proinflammatory milieu that develops with persistent hyperlipidemia (Boden et al., 2005). Recently, studies have suggested that PKCδ may play a role in lipid balance in rodents and humans. Either whole body or liver specific loss of PKCδ decreases liver lipids (whole body knockout mice were also lighter) and improves glucose tolerance while adenoviral-mediated overexpression promotes hepatic steatosis and worsens glucose tolerance (Bezy et al., 2011; Frangioudakis et al., 2009). The effects of PKCδ may be mediated through altered lipogenesis; expression of key lipogenic enzymes was decreased in PKCδ knockout mice and increased following adenoviral overexpression. Hepatic PKCδ mRNA expression was related to higher plasma triglyceride and glucose concentration in humans as well (Bezy et al., 2011). It is possible that its association with insulin resistance ultimately may reflect its ability to regulate ectopic lipid accumulation. Thus, while PKCδ is important for the development of hepatic steatosis the exact role, either as part of an inflammatory signal or a mediator of lipid homeostasis, remains to be defined (Figure 3).

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How exactly do these enzymes impair insulin signaling? Early studies, using phorbol acetates as a PKC activator, suggested that PKC's interfere with insulin receptor activation (Anderson and Olefsky, 1991; Pillay et al., 1990; Takayama et al., 1988). Though putative PKC phosphorylation sites had been identified on the insulin receptor (Coghlan et al., 1994; Lewis et al., 1990), subsequent studies using phosphospecific antibodies failed to identify insulin receptor phosphorylation at these sites in skeletal muscle biopsies obtained from diabetic patients (Kellerer et al., 1995). Yu et al. found that PKCθ activation associated with decreased insulin-stimulated IRS-1 tyrosine phosphorylation was associated with increased IRS-1 serine phosphorylation and recent studies have shown that PKCθ can phosphorylate IRS-1 on Ser 1101, which has been shown to block insulin-stimulated IRS-1 tyrosine phosphorylation (Li et al., 2004). Recent studies have also found that nPKC-mediated impairment of insulin receptor kinase activity may occur in the liver. In rat liver, PKCε and the insulin receptor are closely associated with each other. Moreover, antisense oligonucleotide (ASO)-mediated inhibition of PKCε expression prevents high-fat diet-induced impairments in insulin receptor kinase activation. Together, these data provide a paradigm to explain insulin resistance in both muscle and liver with lipid excess; namely that diacylglycerol-mediated activation of nPKC's directly impair insulin signaling and insulin action.

Ceramides and Akt2

In several of the models discussed previously, the development of insulin resistance is associated with tissue accumulation of ceramide species as well as diacylglycerol, though increases in ceramides may be specific to particular types of lipid challenges (e.g. palmitic acid but not oleic acid). Accumulation of ceramides has been associated with impaired Akt2 action. This appears to be due to a direct effect on Akt2 activation and not an impairment in upstream signaling events (e.g. IR kinase activation or PI3 kinase activation) (Schmitz-Peiffer et al., 1999; Stratford et al., 2004). There are several proposed mechanisms whereby ceramides may impair Akt2 activation. First, ceramides may lead to activation of protein phosphatase 2A (Teruel et al., 2001), which can dephosphorylate Akt2, effectively dampening insulin signaling (Figure 3). In addition, ceramides may impair insulin action via the atypical PKC isoform, PKCζ (Powell et al., 2003). PKCζ and Akt2 interact intracellularly but dissociate upon insulin stimulation; ceramides impair this disassociation and furthermore via PKCζ phosphorylation of Akt2 prevent Akt2 activation (Figure 3). Ceramides may accumulate in caveolin-enriched microdomains (Fox et al., 2007) and recruit PKCζ, which in turn could sequester Akt2 and prevent it from participating in insulin signaling (Blouin et al., 2010). To date, these cellular mechanisms have largely been studied using cell systems and future studies are required to translate these *in vitro* findings to *in vivo* models of insulin resistance and humans.

Lipids and the Unfolded Protein Response

A second broad theme for the pathogenesis of insulin resistance, particularly for hepatic insulin resistance with NAFLD, is the activation of the unfolded protein response (UPR), also termed endoplasmic reticulum stress. The UPR is initiated with the accumulation of unfolded proteins with the ER lumen. Exposure of hydrophobic β-sheets, that should comprise the core of a folded protein, avidly recruit glucose-regulated protein 78 (GRP78, also termed BiP or immunoglobulin binding protein) away from the key UPR effectors allowing activation. Activation of three canonical arms, inositol requiring enzyme-1 (IRE1α), PKR-like ER kinase (PERK) and activating transcription factor-6 (ATF6) work to reduce unfolded proteins with the ER lumen, both by increasing membrane biogenesis (specifically via the IRE1 arm), halting protein translation (via the PERK arm) and enhancing expression of ER chaperones (Okada et al., 2002). Activation of the UPR was observed in the livers of leptin-deficient *ob/ob* mice (Ozcan et al., 2004), suggesting that

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these pathways may be involved in the pathogenesis of insulin resistance in obese states in rodents as well as in humans (Gregor et al., 2009). Chemical inducers of the UPR impaired insulin signaling (Ozcan et al., 2004), whereas chemical chaperones that reduce ER stress improved insulin signaling (Ozcan et al., 2006). And, markers of the UPR are decreased after surgical induced weight loss (Gregor et al., 2009). While the UPR is clearly associated with insulin resistance there are critical gaps in our understanding of this relationship.

In many instances, activation of the UPR provides cells the ability to adapt to changing demands. In insulin resistant states, beneficial adaption is seen in pancreatic β-cells. The increased requirements for insulin production could overwhelm the capacity of the ER to process and secrete insulin. Inhibition of the UPR could lead to β-cell dysfunction, as is seen in β-cell specific XBP1 knockout mice (Lee et al., 2011a). However, in other tissues, such as liver and adipose, activation of the UPR is thought maladaptive; in the prevailing view, IRE1 mediated activation of the Jun-N terminal kinase 1 (JNK1, also known as mitogen activated protein kinase-8, MAPK8) leads to serine phosphorylation of insulin receptor substrate-1 at a key serine (307) which is thought to lead to impaired insulin signaling (Ozcan et al., 2004; Tuncman et al., 2006). Though compelling, several critical questions remain unanswered regarding this paradigm.

The UPR in NAFLD and Hepatic Insulin Resistance

A significant amount of work has focused on the role of UPR in the pathogenesis of hepatic insulin resistance and NAFLD (Figure 3). The UPR regulates lipogenesis, allowing for expansion of the ER membrane and increasing the capacity of the ER to handle proteins. This can be demonstrated even in wild type mice, where chemical activators of the UPR can lead to a transient hepatic steatosis. Genetic mouse models have provided additional insights into the mechanisms whereby the UPR may regulate lipogenesis. Overexpression of GRP78 can suppress activation of the UPR and in *ob/ob* mice lead to a reduction in liver lipid content (Kammoun et al., 2009). This is attributed to a decreased expression in SREBP1c and ChREBP, two key transcriptional regulators of lipogenesis. Mice with liver specific reduction of IRE1α ($IRE1\alpha^{Hepfe/-}$) have minimal phenotypic changes, other than the inability to splice XBP1 into XBP1s and a reduction in ER content (Zhang et al., 2011a). When these mice are treated with chemical activators of the UPR (tunicamycin or bortezomib), they develop a greater degree of hepatic steatosis. These mice displayed enhanced activation of the other UPR arms (PERK-eIF2α phosphorylation and ATF6 cleavage), and the induction of key transcriptional regulators of lipogenesis such as C/EBPβ and δ, LXRα and ChREBP and activation of genes that promote cellular lipid accumulation (e.g. ACC1, DGAT1, DGAT2 and adipose differentiation-related protein, or ADRP, a protein that stabilizes lipid droplets) as well as a decrease in hepatic lipid export. While these changes may be due to increased activation of PERK and ATF6, it is also possible that IRE1α may act to suppress lipid accumulation. Thus, loss of the inhibitory action of IRE1α may allow increased lipid accumulation. Similarly, mice lacking ATF6 α ($ATF6\alpha^{-/-}$) are phenotypically normal, but when challenged with tunicamycin develop hepatic steatosis (Yamamoto et al., 2010). This phenotype was attributed to both a decreased expression of key genes that regulated hepatic lipid oxidation and a marked increase in ADRP. These models demonstrate both that deletion of single arm of the UPR can lead to increased signaling via the unaffected arms and also illustrate the intimate relationship between the UPR and several facets of cellular lipid balance (e.g. lipogenesis, oxidation, etc.).

The PERK arm of the UPR serves to halt protein translation, essentially by inactivating eukaryotic translation initiation factor 2α (Ron and Walter, 2007). Genetic deletion of PERK causes severe growth retardation thought due to marked reduction in hepatic IGF-1 (Li et al., 2003). Interestingly, restoration of PERK in the β-cells alone can provide some degree of rescue; neonatal mice show similar degrees of growth retardation as $PERK^{-/-}$ but

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manifest a "catch up" as juveniles associated with an increase in plasma IGF-1 concentrations. This resonates with the other studies demonstrating that activation of the UPR can be adaptive in the β-cells. Currently, there are no reports of liver or adipose specific deletions in PERK. However, liver-specific inhibition of the PERK pathway was achieved by transgenic expression of the C-terminal fragment of GADD34/PPP1R15a under an albumin promoter (Alb:GC). This gene encodes a phosphatase that specifically dephosphorylates eIF2α, effectively terminating the signal from PERK. Alb:GC mice had reduced body weight and, in contrast to the studies in $IRE1\alpha^{Hepfe/-}$ and $ATF6\alpha^{-/-}$ mice, reduced liver triglyceride content when challenged with ~4 months of high-fat feeding. This was attributed to an impaired transcriptional induction of many key genes associated with the development of hepatic steatosis (e.g. PPARγ, FASn, SCD1, etc.) in Alb:GC mice. As expected, glucose tolerance was improved in these mice.

Alb:GC mice are prone to fasting hypoglycemia and have decreased expression of gluconeogenic enzymes, the latter finding attributed to decreased expression of C/EBPα and β (Oyadomari et al., 2008). Additional metabolic characterizations were recently performed in these mice after three-days of high fat feeding (Birkenfeld et al., 2011). This short dietary challenge can specifically result in hepatic triglyceride accumulation and hepatic insulin resistance without obesity. Whole body calorimetry and body composition analysis did not uncover any differences in energy balance or adiposity, in the Alb:GC mice. The rate of endogenous glucose production was reduced in the Alb:GC mice, consistent with a reduction in fasting plasma glucose concentration. But, these studies also yielded two surprising findings. First, insulin signaling may activate components of the UPR. Increased Grp78 protein expression and XBP1s and CHOP mRNA expression was observed in both wild-type and Alb:GC animals under hyperinsulinemic-euglycemic conditions and the increase in XBP1s and CHOP was enhanced in the Alb:GC mice. Thus, these studies demonstrate again how impairment of a single UPR arm can lead to increased activation of other arms. Secondly, insulin-stimulated muscle and adipose glucose uptake was actually decreased in the Alb:GC mice though neither muscle lipid content nor adiposity was altered. The development of insulin resistance was associated with an increase in hepatic insulin like growth factor binding protein 3 (IGFBP3), which itself can impair insulin action (Yamada et al., 2010). These studies suggest an additional nuanced role of hepatic UPR, the regulation of peripheral insulin action through secreted factors.

The specific role of XBP1s in mediating insulin resistance has also been tested. Mice lacking one copy of XBP1 ($XBP1^{+/-}$) were prone to glucose intolerance and impaired insulin signaling when placed on a high-fat diet (Ozcan et al., 2004). Though this was associated with increased activation of PERK and JNK1, the $XBP^{+/-}$ mice also gained more weight, a significant confounder. In contrast, liver specific deletion of XBP1s (ΔXBP1s) reduced hepatic and plasma lipid concentrations in mice, associated with reduced expression of lipogenic enzymes (Lee et al., 2008). The reduction in lipogenesis is more evident when the mice are challenged with a potent lipogenic stimulus such as a high-fructose diet. Fructose-fed ΔXBP1s have similar body weights and fat mass as their wild-type counterparts. However, in ΔXBP1s are protected from the increases in hepatic diacylglycerol and triglyceride content and they have a reduction in hepatic PKCε activation (Jurczak et al., 2011). And, as with other models, there was evidence for activation of the UPR, though in this model, this did not lead to hepatic steatosis or insulin resistance. Taken together, these studies suggest that the UPR is intricately tied to lipogenesis and that disruption of any one arm may lead to compensatory increases in signaling via the other arms. The ability of the UPR to cause hepatic insulin resistance may ultimately depend on whether UPR activation alters the balance of lipogenesis and lipid export to promote hepatic lipid accumulation.

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The UPR and Insulin Action in Other Tissues

In adipose tissue, UPR activation appears to regulate energy balance. Mice with a heterozygous deletion of Grp78 (*Grp78*$^{+/-}$) had an activation of "adaptive" UPR factors (e.g. C/EBP Homologous Protein (CHOP), XBP1s and EDEM), are protected against diet-induced obesity, hepatic steatosis and insulin resistance (Ye et al., 2010). *Grp78*$^{+/-}$ mice have increase energy expenditure relative to fat-fed wild-type mice, which may account for many of the observed changes. The mechanism for increased energy expenditure is unclear, though increased adipose expression of peroxisome proliferator gamma coactivator 1-α (PGC1α) raises the possibility of increased mitochondrial biogenesis. Thus activation of UPR following loss of the Grp78 may alter adipogenesis and energy balance to improve insulin sensitivity. However, in contrast, *in vitro* studies in 3T3L1 adipocytes suggest that activation of the UPR may increase adipose resistin production, in a CHOP dependent manner (Lefterova et al., 2009) and that the IRE1-XBP1s pathway is critical for adipogenesis (Sha et al., 2009).

Though the canonical role of the UPR in regulating ER function has been largely studied in secretory organs, recent studies are forging a new role for the UPR in skeletal muscle (Figure 2). In mice, chronic high-fat feeding activates the UPR in both early (e.g. 6 weeks high-fat feeding) and late stages (20 weeks). However, 6-weeks of high-fat feeding in healthy human subjects did not activate the UPR in skeletal muscle, even though there were the expected changes in glucose tolerance and intramyocellular lipid accumulation. And recently, the UPR has been shown to interact with PGC1α in exercising muscle. Specifically, PGC1α may co-activate a cassette of genes with ATF6α to induce adaptive changes to exercise.

It is challenging to combine all of the results of the aforementioned studies into a singular mechanistic model to explain how the UPR leads to insulin resistance associated with obesity. The models are varied, with different challenges employed (e.g. varying diets, chemical inducers) and different assessments of glucose and insulin action. Reports that chemical chaperones (e.g. tauroursodeoxycholic acid, TUDCA), improve glucose homeostasis by alleviating ER stress in rodents (Ozcan et al., 2006) and humans (Kars et al., 2010) have been used to buttress the argument that activation of the UPR causes insulin resistance. But these agents can have other, confounding, effects such as suppressing mRNA expression of genes involved in *de novo* lipogenesis (Yang et al., 2010a) and activating deiodinase-2 (DIO2), which can promote thyroid hormone action (da-Silva et al., 2011). Thus it is difficult to conclude that the UPR directly interferes with insulin signaling and lead to insulin resistance. Instead, the data suggest that the UPR functions as a part of integrated cellular response to balance metabolic needs. Some aspects of the UPR clearly regulate lipogenesis (e.g. the IRE1α-XBP1s arm), lipid droplet formation and lipid storage (e.g. through ATF6) and may also regulate glucose metabolism (e.g. signaling through eiF2α and CREBH (Lee et al., 2010; Zhang et al., 2006). Thus, activation of the UPR may primarily alter cellular lipid balance and, via accumulation of lipid intermediates, alter insulin signaling.

Inflammation Integrates a Metabolic Response

Empiric observations demonstrating insulin resistance in septic patients (Clowes et al., 1978; Iochida et al., 1989; Raymond et al., 1981; Shangraw et al., 1989) and later chronic elevation of cytokines in obese and diabetic subjects (Bunout et al., 1996; Pickup and Crook, 1998; Visser et al., 1999; Yudkin et al., 2000) suggested that a pathological activation of the innate immune system may cause insulin resistance and attendant complications, such as atherosclerosis. Rodent studies demonstrated an increase in adipose TNFα in rodent models

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of insulin resistance (Hotamisligil et al., 1993). TNFα expression was increased in the adipose tissue obtained from obese subjects, was related to insulin resistance and, decreased with weight reduction (Hotamisligil et al., 1995; Kern et al., 1995). Though initial studies suggested an impairment of insulin receptor kinase activity, subsequent studies demonstrate that the defect in insulin signaling was attributed to serine phosphorylation of IRS1 at serine-307 residue by activation of jun-N-terminal kinase 1 (JNK1) (Aguirre et al., 2000; Hotamisligil et al., 1996).

Adipose TNFα secretion is now thought to emanate from activated macrophages (adipose tissue macrophages, ATMs) that were recruited via chemokine signaling (Weisberg et al., 2003; Xu et al., 2003). Adipose tissue overexpression of chemokine ligand CCL2 (also known as monocyte chemoattractant protein 1, MCP1) increases ATMs without any change in body weight gain or adiposity (Kanda et al., 2006). These mice exhibit both peripheral and hepatic insulin resistance; the latter is associated with hepatic steatosis. Consistent with this model, deletion of CCL2 protects from high-fat diet induced insulin resistance and is associated with a reduction in hepatic lipid content (Kanda et al., 2006). Deletion of the CCL2 receptor, C-C motif chemokine receptor 2 (CCR2), also decreased ATM accumulation and led to decreased adiposity and body weight. However, even when weight-matched animals were compared, loss of CCR2 (as well treatment with a CCR2 antagonist) still conferred metabolic improvements, as reflected by lower fasting plasma insulin concentrations and improved glucose tolerance (Weisberg et al., 2006). These data suggest that recruitment of ATMs are associated with ectopic lipid accumulation and insulin resistance.

Numerous studies have demonstrated that cytokines increase adipose lipolysis (Kawakami et al., 1987; Stone et al., 1969), but the underlying mechanism is unclear. Earlier studies examined the possibility that cytokines may regulate expression of key lipases. However, TNFα actually decreases expression of ATLG, which is thought to be secondary to decreased PPARγ expression (Kim et al., 2006; Li et al., 2009). IL-6 infusion in healthy humans increases plasma fatty acid and glycerol concentrations (indirect measures of lipolysis). Though adipose mRNA expression of HSL increased with IL-6, there were no changes in protein or HSL activity, suggesting that other mechanisms are responsible (Watt et al., 2005). Recent studies have suggested that cytokines may affect the stability of lipid droplets in adipocytes by influencing the proteins that stabilize the lipid droplet. TNFα decreases perilipin expression, presumably enhancing the ability of lipases to access triglyceride within the lipid droplets (Bezaire et al., 2009; Laurencikiene et al., 2007). TNFα, as well as IL-1β and IFNγ, have also been shown to decrease expression of a newly described lipid droplet protein that is fat specific protein 27 (FSP27, also known as cell death-inducing DFFA-like effector c or CIDE C) (Ranjit et al., 2011). This protein is homologous to perilipin and may also serve to stabilize lipid droplets by controlling lipase access to triglyceride. Silencing of FSP27 enhances basal and TNFα mediated lipolysis while overexpression of FSP27 abbrogates lipolysis. Thus adipose tissue inflammation may lead to macrophage recruitment, which could increase lipolysis. This in turn may also contribute to insulin resistance; excess lipid delivery could promote ectopic lipid accumulation leading to the associated impairments in insulin signaling. But, again, there are subtleties to this paradigm. Lipolysis may also occur with fasting. Calorie restriction in fat-fed mice acutely increases ATMs and may represent a response to the initial increase ATGL mediated lipolysis (Kosteli et al., 2010). Chemical depletion of ATMs with clodronate increases adipose lipolysis, suggesting that ATMs may play a role in regulating the rate of adipose lipolysis. Thus, ATMs, may meter lipid release, and in doing so, prevent excess lipid delivery that would further enhance ectopic lipid accumulation and exacerbate insulin resistance. The interaction between macrophages and adipose tissue may also optimize

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energy usage in the organism, permitting lipid release that can be coordinated with lipid utilization.

ATMs secrete cytokines to orchestrate these metabolic changes in target tissues through a complex series of signaling pathways. For example, TNFα binding to the TNF receptor can lead to activation of the mitogen activated protein kinase pathways (e.g. JNK1, discussed below) as well as the inhibitor of nuclear factor κ-B kinase pathway (IKK), which itself is composed of three subunits: α (IKK1), β (IKK2), and γ (NEMO). Genetic rodent models provide some insights into how this pathway may modulate insulin action. Liver specific deletion of IKK (i.e. $Ikbkb^{\Delta hep}$), essentially blocks signaling through this pathway. $Ikbkb^{\Delta hep}$ mice were protected from hepatic insulin resistance and with improved hepatic insulin signaling in both fat-fed and *ob/ob* mice, though without any improvements in insulin stimulated whole body glucose disposal (Arkan et al., 2005). Deletion of IKK-β in myeloid cells (i.e. $Ikbkb^{\Delta mye}$) conferred greater protection from high-fat diet induced insulin resistance with improvements in both the liver and muscle (Arkan et al., 2005). While these results suggest that action of IKK-β in myeloid cells may lead to insulin resistance, $Ikbkb^{\Delta mye}$ mice were noted to gain less weight than floxed counterparts (though, this difference was not significant). Tissue lipid content was not assessed in these studies. Instead, the improvements seen in these models were attributed to decreased expression of key downstream cytokines (e.g. IL-1, IL-6), though the actual changes in circulating cytokines were only modestly detected for IL-1β.

Full IKK action requires the regulatory γ-subunit NF-kB essential modulator (NEMO). Liver-specific deletion of NEMO ($NEMO^{L\text{-}KO}$) will prevent IKK activation and suppress signaling via this pathway. Surprisingly, $NEMO^{L\text{-}KO}$ have several hallmarks of increased inflammation, with increased expression of hepatic cytokine mRNA (e.g. TNFα and IL-6) and increased activation of JNK1 (Luedde et al., 2007). When placed on a high-fat diet, $NEMO^{L\text{-}KO}$ mice gain less weight than wild-type counterparts yet develop marked steatohepatitis. The latter was associated with a mismatch between the response of PPARα and PPARγ to high-fat feeding. PPARα was not induced while PPARγ had greater induction; the net result is a shift in the transcriptional regulation that decreases lipid oxidation and favors lipogenesis. Defying the expectations stemming from increased inflammation and hepatosteatosis, fat-fed $NEMO^{L\text{-}KO}$ have lower fasting plasma glucose and insulin concentrations and improved glucose tolerance (Wunderlich et al., 2008). The explanation for the improvement in glucose metabolism is not entirely clear. Key lipid intermediates were not assessed. Instead, the improvements were attributed to decreased expression of key gluconeogenic enzymes (PEPCK and glucose 6-phosphatase).

NF-κB is the classic IKK target. Phosphorylation of the IκBα subunit of the NF-κB complex by IKK leads to proteasomal degradation, allowing nuclear translocation of the p50 and p65 subunits. Overexpression of the p65 subunit will partly simulate NF-κB activation. Adipose specific expression of p65 (aP2-p65) activates adipose specific inflammation (ATM recruitment, cytokine production, etc.) but leads to small adipose pads and protects mice from weight gain when placed on a high-fat diet (Tang et al., 2010). This is attributed to an increase in energy expenditure. Accordingly, ap2-p65 transgenic mice are protected from diet-induced insulin resistance, with improved hepatic and peripheral insulin sensitivity; the latter was due to an increase in insulin-stimulated glucose uptake in WAT and heart, but not skeletal muscle. The p50 subunit can antagonize the effects of p65. Mice lacking p50 (p50-KO) exhibit a similar phenotype as the aP2-p65 mice, with increased inflammation, increased energy expenditure and decreased adiposity. The Iκ-B kinase ε (IKKε) was recently described as a kinase whose expression is increased by NF-κB activation. Mice lacking IKKε exhibit a marked upregulation of adipose UCP1 expression associated with an

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in energy expenditure with protection from diet-induced adiposity and insulin resistance (Chiang et al., 2009).

Activation of inflammatory pathways has been observed as part of a normal physiological response. In response to acute aerobic exercise, normal subjects and obese, non-diabetic subjects had an increase in skeletal muscle cytokine expression (MCP1 and IL-6) as well as activation of the NF-κB pathway (Tantiwong et al., 2010). Subjects with T2DM had high basal NF-κB activity but did not mount an incremental increase with exercise. The elevations in IL-6, also considered a myokine, may promote muscle lipid oxidation. In normal, healthy males, infusion of IL-6 led to increases in both lipolysis and lipid oxidation across the femoral vascular bed (Wolsk et al., 2010). Though this study had some methodological concerns (e.g. lipolysis from subcutaneous adipose tissue could have occurred, rates of lipid oxidation are dependent on numerous assumptions, etc.), these studies do recapitulate earlier studies demonstrating that IL-6 was capable of increasing lipid oxidation in isolated rat soleus (Bruce and Dyck, 2004). As with many of these pathways, physiologically appropriate regulation is critical. Inappropriate activation of the IKK/NF-κB pathway, as seen in mice with muscle specific overexpression of IKK-β (MIKK mice), can lead to muscle atrophy (Cai et al., 2004). In the MIKK mice, chronic, forced activation of this pathway leads to increased energy expenditure, which was associated with both increased rates of both protein synthesis and breakdown. These studies identified the E3 ubiquitin ligase muscle RING finger protein 1(*MuRF1*) as a novel target of NF-kB, that is upregulated in the MIKK mice and may account for the futile protein cycling that leads to muscle atrophy.

These studies seem to offer discordant conclusions. Some models in which inflammatory pathways are activated (e.g. ap2-p65, p50-KO, MIKK) have increased energy expenditure. In contrast, deletion of IKKε, which represents a selective block in inflammatory signaling, also increases energy expenditure. Resolving these differences on a mechanistic basis is not trivial. The immune system was intended to coordinate whole body responses to various stimuli (Figure 4). Inferring the role of innate immunity in the development of insulin resistance based on genetic manipulations of single proteins in specific tissues is challenging. However, in these disparate models, there is a consistent axiom. Models with increased energy expenditure are protected from weight gain, ectopic lipid accumulation and insulin resistance.

Rethinking the Role of JNK in Insulin Resistance

Despite the multiplicity of intracellular pathways that mediate the “inflammatory responses,” the explanation for the development of insulin resistance in inflammatory states often converges on the activation of JNK1. Activation of JNK1 also plays a prominent role in the mechanism whereby the UPR (via IRE1α activation) may cause insulin resistance. In inflammatory signaling pathways (e.g. in response to TNFα), JNK1 activation follows a parallel pathway to NF-κB activation. JNK1 knockout mice were shown to be protected from diet-induced insulin resistance. However, JNK1 knockout mice were also noted to gain less weight compared to their wild-type control littermates and had slightly higher body temperatures, implying an increase in energy expenditure (Hirosumi et al., 2002). These changes were also seeing following JNK1 inactivation in macrophages and adipocytes (via transgenic expression of a dominant negative form of JNK1 under the aP2 promoter) (Tuncman et al., 2006). These mice had an increase in energy expenditure and were protected from diet-induced obesity, hepatic steatosis and glucose intolerance. Similar effects are seen in *ob/ob* mice treated with an ASO against JNK1. JNK1 ASO increased energy expenditure, possibly via increased BAT expression of UCP1, decreased body weight gain and liver triglyceride content, decreased PKCε activation and improved insulin

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sensitivity. Thus, as in the previous models where intracellular cytokine signaling is impaired (e.g. IKKε knockout mice), deletion of JNK1 can lead to increases in energy expenditure, decreased accumulation of ectopic lipids and protection from insulin resistance.

Tissue specific manipulations of JNK1 provide additional insights. Liver specific JNK1 deletion actually promoted hepatic steatosis, associated with increased expression of key lipogenic enzymes, impaired insulin signaling and hepatic insulin resistance (Zhang et al., 2011b). Unexpectedly, insulin clearance was increased, possibly from increased expression of the insulin receptor and CECAM-1. Similar findings were observed with adenoviral-mediated decrease of hepatic JNK1, notably an increase in liver lipid production and a decrease in fasting plasma insulin concentrations (Sabio et al., 2009). While there are no published reports of tissue specific JNK1 activation, some hints can be gleaned from mice lacking mitogen activated kinase phosphatase-1 ($MKP1^{-/-}$), a phosphatase that inactivates MAPKs, such as JNK1 (Wu et al., 2006). In $MKP1^{-/-}$ mice, JNK1 activity is increased, (as is the activity of erk2 and p38-MAPK) specifically in the nucleus. Despite the increased JNK1 activity, these chow fed $MKP1^{-/-}$ mice have normal hepatic insulin sensitivity. In counterpoint to the changes in the liver specific JNK1 knockout mice, $MKP1^{-/-}$ mice have evidence of increased lipid oxidation and decreased lipogenesis. This latter finding may be due to MAPK mediated inhibition of PPARγ in $MKP1^{-/-}$ mice (Flach et al., 2011). These studies consistently point to a role of JNK1 in regulating hepatocellular lipid balance, possibly through a combination of inhibiting lipogenesis and increased energy expenditure leading to increased lipid oxidation.

Additional tissue specific deletions of JNK1 complete the variations of this theme. Adipose JNK1 knockout mice are protected from hepatic steatosis and specifically hepatic insulin resistance though they exhibit similar weight gain and adiposity on a high-fat diet (Sabio et al., 2008). This protection was attributed to decreased adipose release of IL-6, which is thought to impair insulin action via SOCS3. More recently, muscle specific JNK1 knockout were studied (Sabio et al., 2010). Fat-fed muscle specific JNK1 knockout mice have similar weight gain and impairment of glucose tolerance as wild type mice. However, under hyperinsulinemic-euglycemic clamp conditions, they show a modest improvement in muscle specific glucose uptake with improvement in insulin signaling. Surprisingly, these mice also manifest increased adipose tissue inflammation and modest increase in hepatic steatosis, though without differences in adipose or hepatic insulin sensitivity.

These studies portray a nuanced role of JNK1 in the pathogenesis of insulin resistance, with different effects in different tissues. Placing the role of JNK1 activation within a comprehensive physiological framework will be challenging due to the high degree of interconnectedness spanning various signaling networks. For example, JNK1 activation is thought to induce insulin resistance by serine phosphorylation of IRS1 on a critical serine 307 residue. But insulin signaling itself may also promote phosphorylation of serine 307, independent of the TNFα-JNK1 pathway (Rui et al., 2001). In contrast, recent studies suggest that serine 307 phosphorylation may actually augment insulin action (Copps et al., 2010). While we can conclude that alterations in JNK1 activity can alter insulin responsiveness, its role may be indirect and instead be related to its ability to alter intracellular lipid metabolism. Specifically, activation of JNK1 in adipocytes may increase cytokine release that could be associated with increased lipolysis. Activation of JNK1 in hepatocytes (presumably from increased cytokines) may suppress lipogenesis. This coordinate activation of JNK1 in key tissues may be part of an orchestrated move to shift metabolic fuel substrates.

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Translation to the Bedside

The studies in rodent models are invaluable for understanding the potential cellular mechanisms for the pathogenesis of insulin resistance. But, are these pathways relevant to humans? Recently, Kumashiro *et al.* studied a cohort of obese, but non-diabetic subjects who were undergoing bariatric surgery and assessed each of these pathways in flash-frozen liver biopsies (Kumashiro et al., 2011). Hepatic DAG strongly related to insulin resistance as assessed by the homeostatic model of insulin resistance (HOMA-IR) (much better so than body mass index) and activation of PKCε. Though PKC activation is typically marked by membrane translocation, plasma membrane DAG content did correlate with insulin resistance. Instead, the DAG concentration of a lipid droplet containing cytoplasmic fraction was the best predictor of insulin resistance in these individuals. In contrast, hepatic ceramide content did not relate to insulin resistance. Moreover, there was only a partial activation of the unfolded protein; specifically, insulin resistance related to eIF2α phosphorylation and CHOP protein expression, both of which are downstream of PERK while other aspects of the UPR did not relate to insulin resistance, including activation of JNK1. Finally, there was no relationship between plasma cytokine concentration (TNFα, IL-1β, IL-6 and CRP) and hepatic cytokine expression and insulin resistance, suggesting that, in this cohort of patients, inflammation did not contribute to the development of insulin resistance.

Teological and Clinical Implications

In trying to integrate these mechanisms into a single model, it is may be useful to consider the roles that these three pathways may have played in evolution. After all, these pathways originally arose to the advantage of the organism; to allow it to adapt to and thrive in differing environments. The unfolded protein response may be the most ancient, present in yeast, the worm and fly and mammals. This pathway may allow a *cell* to integrate metabolic signals and adapt accordingly. The innate immune system may also serve to alter energy metabolism; in Drosophila, this system can halt growth to allow an *organism* to marshal metabolic resources to defend against a pathogen. These two systems are highly interconnected allowing for a coordinated response to various environmental stimuli. However, the ability of these systems to regulate insulin action may be reliant on their ability to alter the concentration of key lipid signaling intermediates.

These mechanisms that modulate insulin action have persisted throughout metazoan evolution and presumably conferred a survival advantage. They were adaptive in an environment of relative nutrient scarcity that required the ability to shift from glucose oxidation to lipid oxidation. Coordinating this metabolic shift may require the ability to dampen insulin signaling in response to increased lipid delivery. This can be demonstrated in humans. Subjects who undergo a prolonged fast become insulin resistant, with reduced insulin-stimulated glucose disposal under hyperinsulinemic-euglycemic conditions (van der Crabben et al., 2008). The development of insulin resistance is associated with increases plasma fatty acid concentrations, as well as an accumulation in muscle lipid content. Activation of PKCs was also associated with starvation-induced insulin resistance in rodents (Hoeks et al., 2010). Thus, it is plausible that the ability of lipid to trigger insulin resistance may be part of a coordinated response to preserve glucose for the central nervous system and vital glucose-obligate organs during starvation.

The cellular mechanisms discussed above demonstrate how systems that were finely tuned to adapt to a changing environment can become pathogenic in a modern environment of nutrient excess. Evidence presented in this review suggest that ectopic lipid (diacylglycerols and possibly ceramides) may be at the root cause of liver and muscle insulin resistance and that new therapies aimed at decreasing lipid content in these organs will represent the most

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efficacious therapeutic targets for the treatment of insulin resistance and its associated co-morbidities. At a societal level, concerted efforts to restore balance in our diets and behaviors can prevent obesity and ectopic lipid deposition, and allow these ancient pathways to return to their physiological role in a healthy life.

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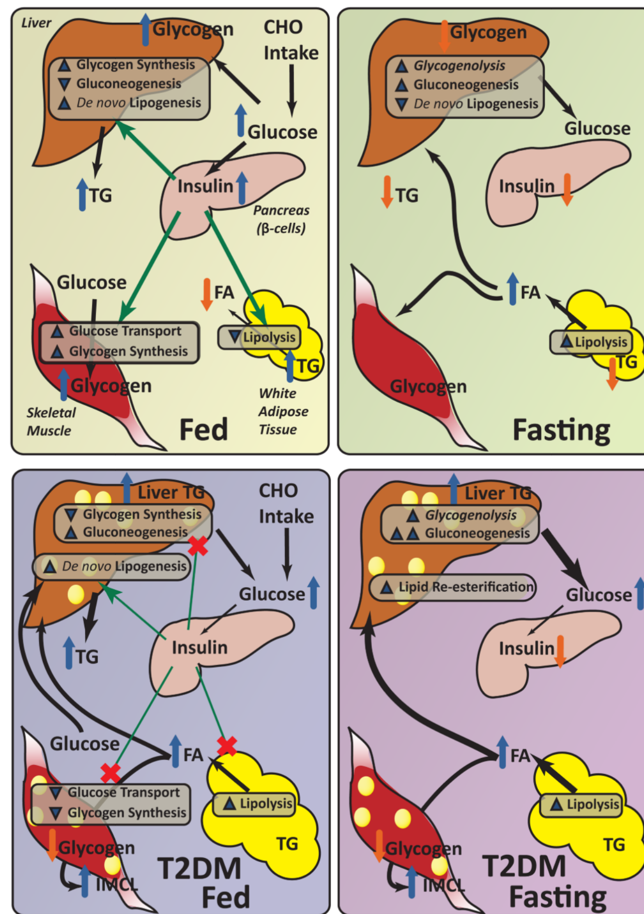

Figure 1. Overview of insulin action

Left Panel: In the fed state, dietary carbohydrate (CHO) increases plasma glucose and promotes insulin secretion from the pancreatic β-cells. Insulin has numerous actions to promote storage of dietary calories, but only several are illustrated here. In the skeletal muscle, insulin increases glucose transport, permitting glucose entry and glycogen synthesis. In the liver, insulin promotes glycogen synthesis and *de novo* lipogenesis while also inhibiting gluconeogenesis. In the adipose tissue, insulin suppresses lipolysis and promotes lipogenesis. **Middle Panel:** In the fasted state, insulin secretion is decreased. The drop in insulin (as well as the action of other hormones which are not depicted), serve to increase hepatic gluconeogenesis and promote glycogenolysis. Hepatic lipid production diminishes while adipose lipolysis increases. **Right Panel:** In type 2 diabetes, ectopic lipid accumulation impairs insulin signaling (as depicted by the red "x"). With accumulation of intramyocellular lipid (IMCL), insulin mediated skeletal muscle glucose uptake is impaired. As a result, glucose is diverted to the liver. In the liver, increased liver lipid also impairs the ability of insulin to regulate gluconeogenesis and activate glycogen synthesis. In contrast, lipogenesis remains unaffected, and together with the increase delivery of dietary glucose, leads to increased lipogenesis and worsening NAFLD. Impaired insulin action in the adipose tissue allows for increased lipolysis which will promote re-esterification of lipids in other tissues (e.g. liver) and further exacerbates insulin resistance. Coupled with a decline in pancreatic β-cells (depicted by the smaller lines emanating from the pancreas), hyperglycemia develops.

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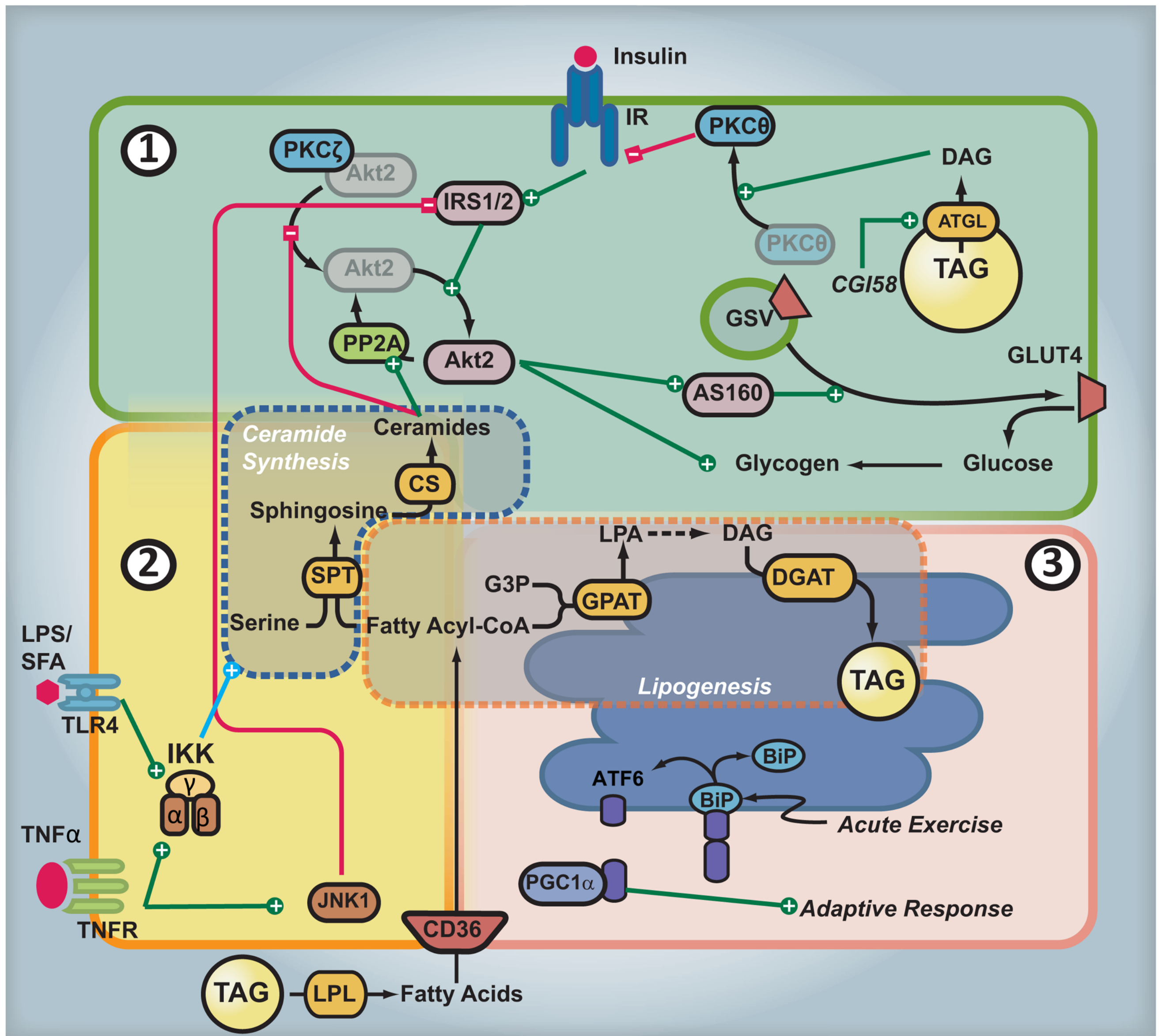

Figure 2. Schematic representation of pathways involved in muscle insulin resistance

Insulin activates the insulin receptor tyrosine kinase which subsequently tyrosine phosphorylates IRS1. Through a series of intermediary steps, this leads to activation of Akt2. Akt2 activation, via AS160 and Rab-GTPase (not shown), promotes the translocation of GLUT4 containing storage vesicles (GSV's) to the plasma membrane permitting the entry of glucose into the cell and promotes glycogen synthesis. This central signaling pathway is connected to multiple other cellular pathways that are designated by numbers 1–3. **1)** The green shaded areas represent mechanisms for lipid induced insulin resistance, notably diacylglycerol mediated activation of PKCθ and subsequent impairment of insulin signaling, as well as ceramide mediated increases in PP2A and increased sequestration of Akt2 by PKCζ. Impaired Akt2 activation limits translocation of GSV's to the plasma membrane resulting in impaired glucose uptake. Impaired Akt2 activity also decreases insulin mediated glycogen synthesis. **2)** The yellow areas depict several intracellular inflammatory pathways, notably the activation of IKK, which may impact ceramide synthesis and the activation of

JNK1, which may impair insulin signaling via serine phosphorylation of IRS1. **3)** The pink area depicts that activation of the UPR which under some instances (e.g. acute extreme exercise) may lead to activation of ATF6 and a PGC1α mediated adaptive response. The ER membranes also contain key lipogenic enzymes and give rise to lipid droplets. Proteins that regulate the release from these droplets (e.g. ATGL and PNPLA3) may modulate the concentration of key lipid intermediates in discrete cell compartments.

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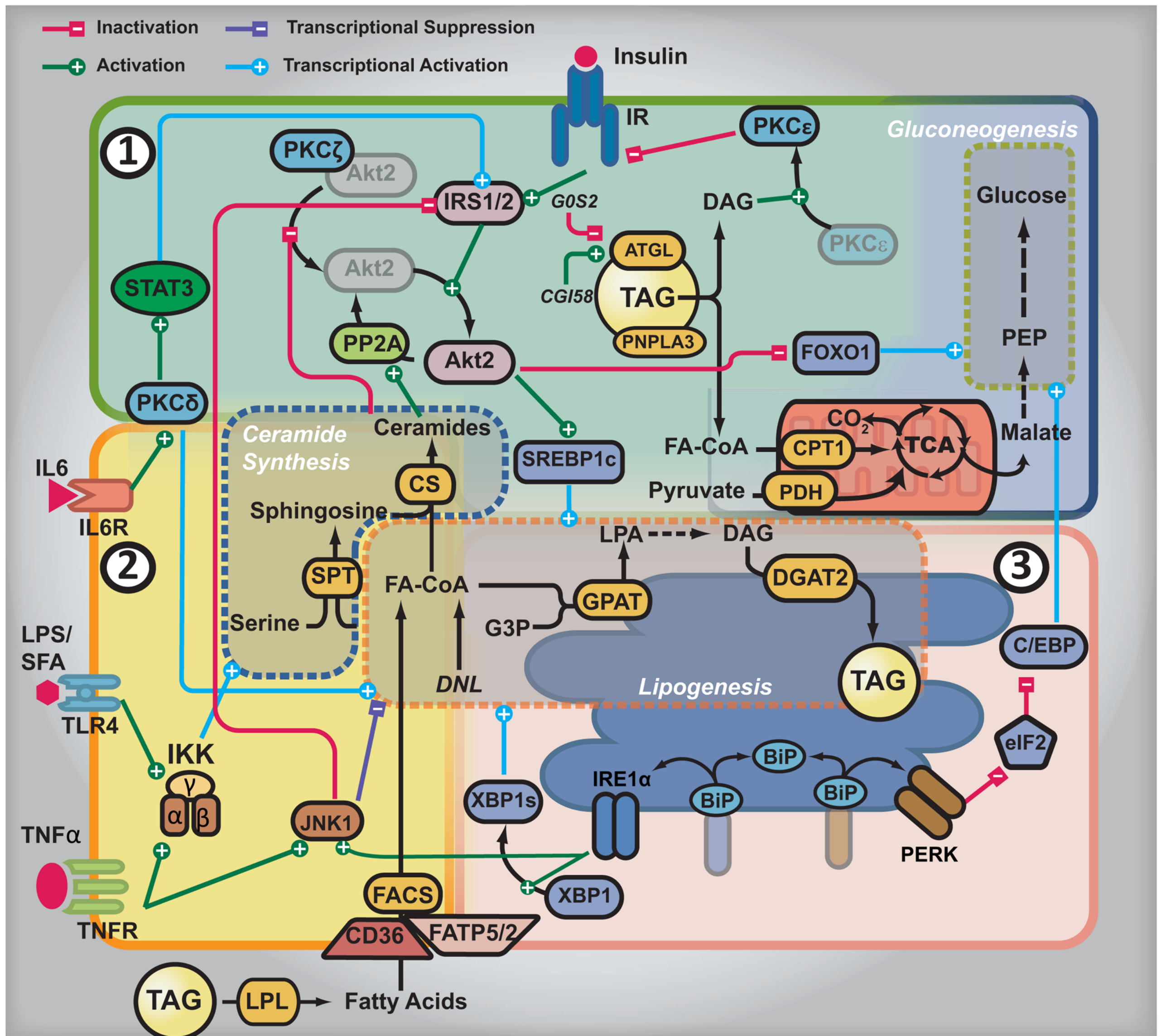

Figure 3. Schematic representation of pathways involved in hepatic insulin resistance

Insulin activates the insulin receptor tyrosine kinase which subsequently tyrosine phosphorylates IRS1 and 2. Through a set of intermediary steps, this leads to activation of Akt2. Akt2 can promote glycogen synthesis (not shown), suppress gluconeogenesis and activate *de novo* lipogenesis (DNL). This central signaling pathway is connected to multiple other cellular pathways that are designated by numbers 1–3. **1)** The green shaded areas represent mechanisms for lipid induced insulin resistance, notably diacylglycerol mediated activation of PKCε and subsequent impairment of insulin signaling, as well as ceramide mediated increases in PP2A and increased sequestration of Akt2 by PKCζ. Impaired Akt2 activation limits the inactivation of FOXO1 and allows for increased expression of key gluconeogenesis enzymes. Impaired Akt2 activity also decreases insulin mediated glycogen synthesis (not depicted). **2)** The yellow areas depict several intracellular inflammatory pathways, notably the activation of IKK, which may impact ceramide synthesis and the activation of JNK1, which may impair lipogenesis. **3)** The pink area depicts that activation of the UPR that can lead to increased lipogenesis, via XBP1s and also increased

gluconeogenesis via C/EBP. The ER membranes also contain key lipogenic enzymes and give rise to lipid droplets. Proteins that regulate the release from these droplets (e.g. ATGL and PNPLA3) may modulate the concentration of key lipid intermediates in discrete cell compartments.

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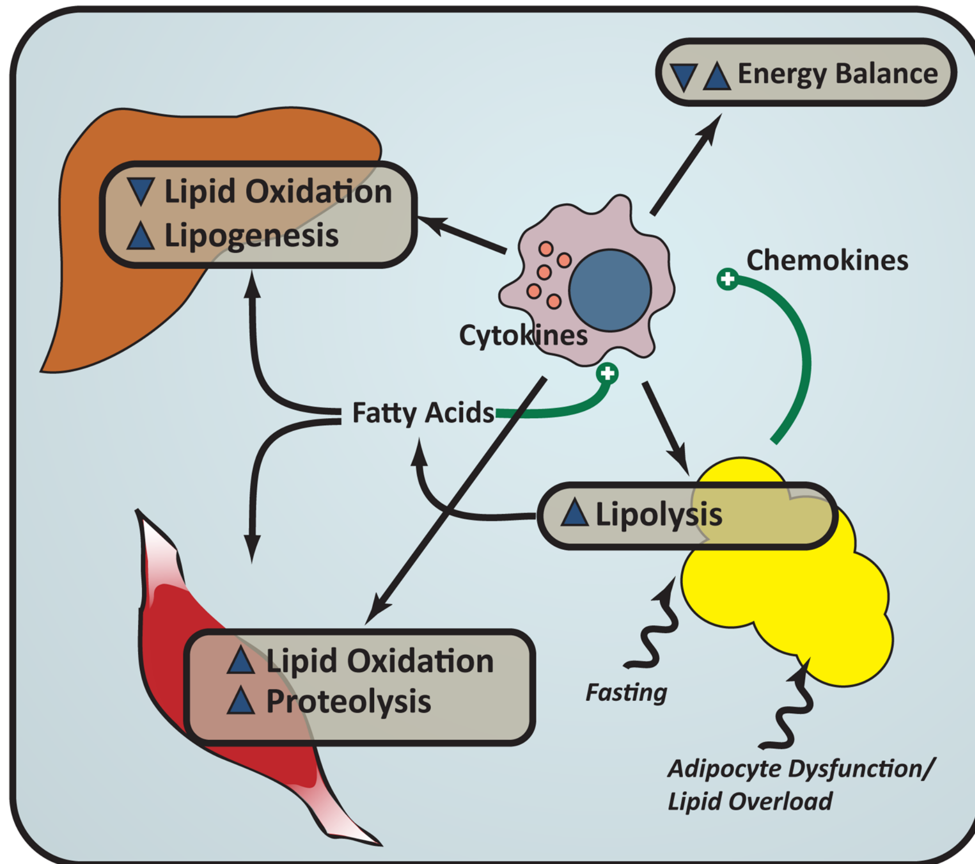

Figure 4. Inflammation and energy metabolism

Under certain conditions (e.g. adipocyte dysfunction or fasting), the release of chemokines and/or lipolysis from adipose tissue promotes macrophage activation. Activated macrophages can then signal to other tissues via the release of various cytokines (e.g. TFNα, IL-6, etc.). In adipocytes, cytokine signaling promotes lipolysis via a decrease in lipid droplet stabilizing proteins (e.g. perilipin or FSP27). In muscle cells, cytokines can promote increase lipid oxidation, and under extreme situations, may promote muscle atrophy via increased proteolysis. In the liver, cytokine signaling may serve to increase lipogenesis and impair lipid oxidation. The effects on energy balance may largely depend on the specific cytokines that are activated and the extent of activation. However, the changes in energy balance may largely dictate the changes in ectopic lipid accumulation and, ultimately the development of insulin resistance.