The Brown Adipose Cell: A Unique Model for Understanding the Molecular Mechanism of Insulin Resistance

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Abstract*:* Type 2 diabetes mellitus (NIDDM) is a complex metabolic disease that occurs when insulin secretion can no longer compensate insulin resistance in peripheral tissues. At the molecular level, insulin resistance correlates with impaired insulin signaling. This review provides new insights into the molecular mechanisms of insulin action and resistance in brown adipose tissue (BAT) and pinpoints the role of BAT in the control of glucose homeostasis.

Keywords: Brown adipocytes, insulin signaling, insulin resistance, insulin receptor, insulin receptor substrates, adipocyte differentiation.

INTRODUCTION

Type 2 diabetes mellitus (NIDDM) is a complex metabolic disease with an environmental and genetic component affecting over 5% of the population in Western societies. NIDDM represents the final stage of long existing metabolic disturbances with deleterious effects on the vascular system, tissues and organs. Consequently, urgent efforts are required to reduce the growing number of patients with this form of a "silent killing" metabolic disease. These efforts include the design of chemotherapeutic compounds to target cellular components or molecular events implicated in the development of the disease. The pathogenesis of NIDDM involves abnormalities in both insulin action and secretion [1]. At the molecular level, insulin resistance, the first detectable defect in Type 2 diabetes, correlates with impaired insulin signaling in peripheral tissues. In addition to muscle and liver, adipose tissues are relevant sites of insulin action. Insulin resistance in adipose tissues leads to an increase of lipolysis with subsequent release of glycerol and free fatty acids (FFA) into the circulation. It is widely accepted that increased availability and utilization of FFA contribute to the development of skeletal muscle insulin resistance as well as to increased hepatic glucose production [2-4]. Consequently, resistance to the anti-lipolytic action of insulin in adipose tissues produces deleterious defects in glucose homeostasis.

Two types of adipose tissue have been described. White adipose tissue (WAT) is specialized to store triglycerides and to release free fatty acids in response to changing energy requirements. In addition, mammals have a second terminally differentiated adipose cell type that composes brown adipose tissue (BAT), which is involved in the dissipation of energy via heat generation. Morphologically, brown adipocytes are characterized by the presence of multiple lipid droplets and a high number of mitochondria [5]. Both white and brown adipocytes are insulin target cells. Whereas insulin resistance in WAT has been extensively studied by a number of laboratories, little is known about the potential role of insulin action or insulin resistance in BAT in Type 2 diabetes.

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FUNCTION AND CHARACTERISTICS OF BROWN ADIPOSE TISSUE

Adipocyte differentiation is a complex process that requires communication between extracellular stimuli in a coordinated network of receptors and transcription factors in the nucleus. While most studies of adipogenesis have focused on the development of WAT, which is the primary site of storage of triglycerides and release of fatty acids in response to changing energy needs [6], little is known about the development of BAT. BAT is a major site for non-shivering thermogenesis in mammals. The unique thermogenic capacity of BAT results from the expression of the uncoupling protein-1 (UCP-1) in the mitochondrial inner membrane required to address the physiological hypothermia in newborn mammals [7]. The function of UCP-1 is to uncouple the proton electrochemical gradient generated by the respiratory chain from ATP synthesis. UCP-1 allows the re-entry of protons and, as a consequence, energy liberated from substrate oxidation is dissipated as heat (Fig. **1**). This mechanism is reversible, since it can be inhibited by purine nucleotides such as GDP [5]. In addition, BAT is a major site for lipid metabolism, fatty acids being the main fuel to maintain the thermogenic capacity of the tissue [8]. In this regard, unlike WAT, BAT does not accumulate lipids simply as a storage depot, but as a source of fatty acids to be oxidized in the mitochondria when BAT thermogenesis is activated in order to produce heat. BAT is activated *in vivo* under particular circumstances such as cold exposure, high fat diet feeding and during the perinatal period as a defense against cold or obesity. Acute activation of BAT thermogenesis appears to be mediated by noradrenaline (NA) liberated from the sympathetic nervous system. By acting through β-receptors, NA increases intracellular cAMP levels, which in turn activate lipolysis (Fig. **1**). The resulting free fatty acids are used as substrates for mitochondrial respiration and as activators of UCP-1 [5]. Brown fat exists

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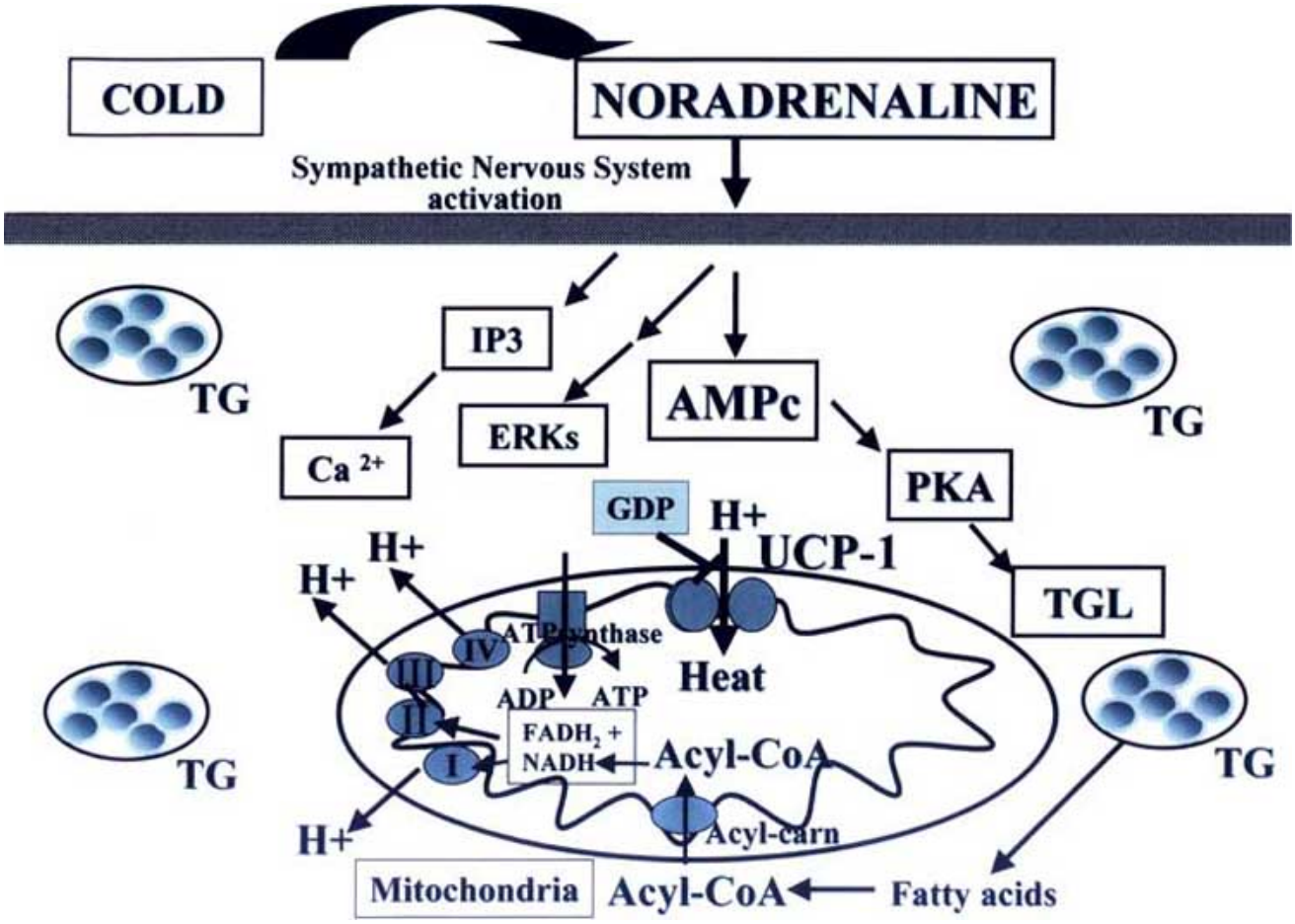

Fig. (1). Mechanism of Cold-induced Thermogenesis: activation of UCP-1. Cold is sensed by the brain, thus activating sympathetic nerves and releasing NA. Acting through different types of adrenergic receptors, NA leads to activation of different signal transduction pathways such as MAPK and cAMP/PKA. PKA phosphorylates triglyceride lipase (TGL) and fatty acids are released from fat depots, which function as activators of UCP-1 and substrates for mitochondrial oxidation generating $NADH.H^+$ extruded by the respiratory chain reenter through UCP-1 instead of through ATP synthase and energy from the proton electrochemical gradient is released as heat.

in the interscapular fat pad of rodents. In humans, there are significant collections of BAT in the neonatal period primarily in the thoracic cavity surrounding the great vessels. Recent data have indicated that in adults white fat contains small islands of BAT and UCP-1 is detectable by PCR techniques [9]. However, in humans the implication of BAT in energy expenditure is still under discussion. The human UCP-1 gene has been cloned, sequenced and mapped to the long arm of chromosome 4 (q31), allowing the identification of several genetic variants. Recently, Mori and co-workers [10] screened the human UCP1 gene for polymorphisms associated with susceptibility to Type 2 diabetes. They found an A/C transition in the 5' untranslated region of exon 1 (112 bp upstream of the translation initiation codon) and also a Met229/Leu variant. Interestingly, the allele frequencies for the C variant and for the Leu229 variant were higher in the Type 2 diabetic group than in the control group. The authors of this study propose that the A/C transition could result in impaired promoter activity and subsequently, in a reduced abundance of UCP-1 protein. Other SNPs have been identified along the human UCP-1 gene [11]. Although none of these are associated with Type 2 diabetes, the frequent A/G substitution at position -3826 have been reported to act synergistically with the β3-adrenergic receptor polymorphism Trp64/Arg increasing the risk of weight gain [12] or endothelial injury [13]. However, an adequate study size will be necessary to confirm these data.

It is well known that in rodents brown adipocytes differentiate at the end of the fetal life on basis of two programs: an adipogenic program related to lipid synthesis and the expression of lipogenic enzymes, resulting in a multilocular fat droplets phenotype, and a thermogenic program related to UCP-1 expression and heat production. Regarding adipogenesis, the expression of the adipogenic-related genes occurs as a multistep process; the fatty acid synthase (FAS) gene (the main lipogenic enzyme) is expressed as early as day 20 of fetal life in the rat. Expression of NADPH-generating enzymes (such as malic enzyme and glucose 6-phosphate dehydrogenase) is initiated mainly on day 21, increasing significantly on day 22 [14]. This induction of lipogenic enzymes correlates with the accumulation of lipids that results in the multilocular fat

droplets phenotype before birth. Thermogenic differentiation of fetal brown adipocytes can be monitored by the expression of UCP-1. UCP-1 mRNA gradually accumulates during the last two days of fetal development, its expression reaching the maximal levels during the first postnatal week [15]. Since the noradrenergic stimulus, induced by hypothermia after birth, it is not yet fully developed in brown adipose tissue during late fetal development [14,16], other potential candidates involved in the onset of differentiation-related gene expression have been implicated. Recently, regulatory elements for triiodothyronine and retinoic acid have been identified in the UCP-1 promoter, suggesting alternative pathways for brown fat thermogenesis [17,18]. In addition, during the last years our laboratory has found that fetal brown adipocytes display high affinity binding sites for insulin and insulin-like growth factor-I (IGF-I) [19,20]. These receptors appear to be the main signals involved in the onset of adipogenic and thermogenic differentiation of brown adipose tissue during late fetal development through their ability to induce the genetic expression of metabolic genes. Additionally, these receptors may have a role in thermogenesis by inducing UCP-1 expression [19,21]. Accordingly, the expression of IGF-I and its receptor in rat BAT during the last days of the fetal period is coincident with an induction of the expression of UCP-1 and several adipogenic-related genes [14]. In addition, it is possible that IGF-I also plays a role in BAT upon cold exposure, since IGF-I mRNA is transiently up-regulated [22].

THE INSULIN/IGF-I SIGNALING CASCADE

Insulin and IGF-I initiate the biological effects in target cells by binding to and activating its endogenous tyrosine kinase receptors [23,24]. These receptors are believed to transduce signals by phosphorylation on tyrosine residues of several cellular substrates including IRS proteins (IRS-1,-2,-3 and –4) [25-28]. These phosphorylated substrates, then, bind proteins containing Src homology 2 (SH2) domains. The p85 regulatory subunit of phosphatidylinositol 3-kinase (PI 3-kinase) contains two SH2 domains that allow its binding to the IRS proteins [29]. Furthermore, the growth factor receptor binding protein 2 (Grb-2), which links signaling via SOS to activation of the Ras complex [30], and protein tyrosine phosphatase SHP-2 [31], that lead to activation of various downstream signaling pathways, are also targets of IRS proteins. PI 3-kinase is a heterodimer composed of an 85-kDa regulatory subunit and a 110-kDa catalytic subunit, capable of phosphorylating phosphoinositides at the 3´-position of the inositol ring, thus initiating phospholipid turnover [29]. The phosphoinositides produced by PI 3-kinase activate the phosphoinositide-dependent kinases 1 and 2. These kinases phosphorylate and activate the serine/threonine kinase PKB/Akt [32]. The mammalian target of rapamycin (mTOR), a 290 kDa serine/threonine kinase and aminoacid sensor, is a direct target for Akt [33]. mTOR can phosphorylate and activate the prototypical p70s6k, a process which is blocked by rapamycin. P70s6k is involved in the phosphorylation of ribosomal protein S6, a constituent of the 40S ribosomal subunit, which mediates the translation of a subgroup of mRNAs encoding ribosomal proteins and elongation factors that are necessary for translation [34].

Grb-2 contains a SH2 domain flanked by two SH3 domains and appears to function as an adaptor molecule coupling growth factor receptors with SOS, a guanine nucleotide-releasing protein that is able to promote the exchange of GTP to GDP on p21 Ras, resulting in the activation of Ras in its GTP state [35]. Ras activation of MAPK cascade has been demonstrated by the fact that RasGTP binds to the amino-terminal cysteine-rich regulatory

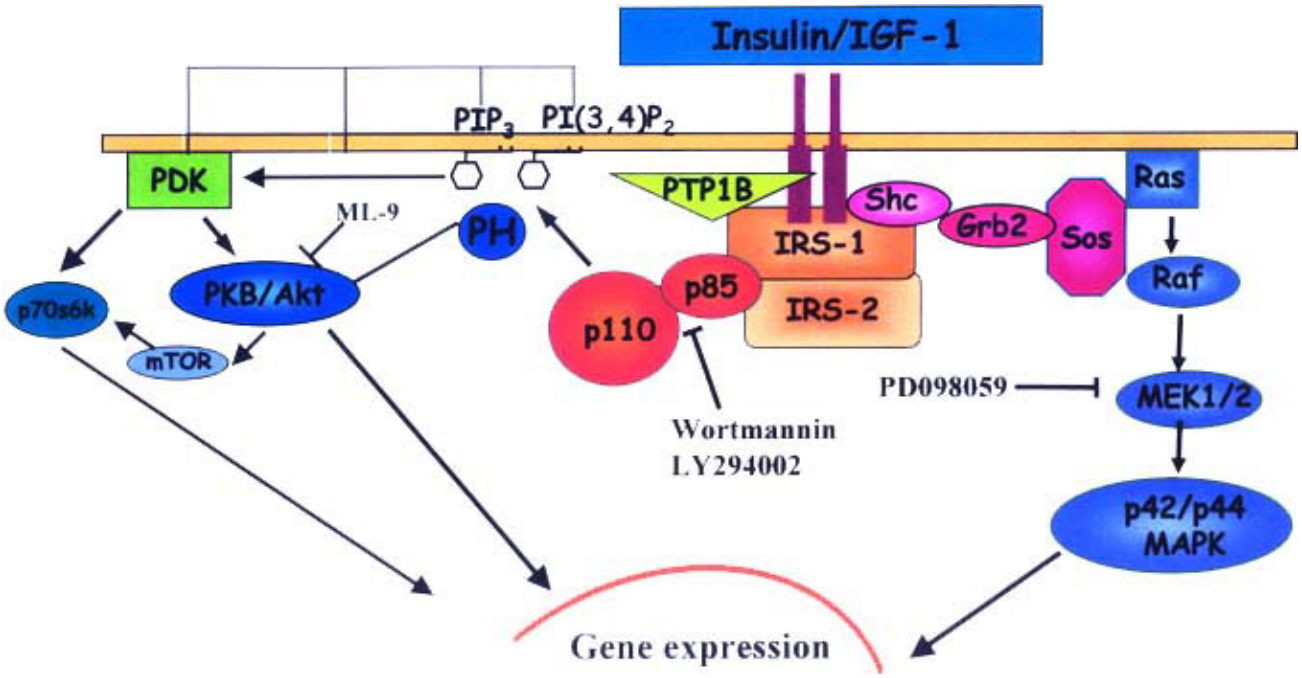

Fig. (2). Insulin signal Transduction Pathways. Insulin and IGF-I activate various signal transduction pathways such as PI 3-kinase and MAPK. The PI 3-kinase pathway is required for differentiation and the Ras/MAPK pathway is necessary for proliferation.

region of the proto-oncogene product Raf-1, a serine/threonine kinase [36]. Then, activated Raf directly phosphorylates MEK, which in turn phosphorylates and activates MAPK [37,38]. One of the well-known downstream targets of MAPK is p90 RSK (ribosomal S6 kinase). Both MAPK and p90 RSK translocate to the nucleus and phosphorylate various transcription factors including c-fos and c-myc [39]. A general schematic depicting the main signal transduction pathways activated by the insulin/IGF-I receptor is shown in Figure **2**.

ROLE OF INSULIN/IGF-I IN BROWN ADIPOCYTE DIFFERENTIATION: MOLECULAR MECHANISMS

Fetal brown adipocytes primary cultures offer an excellent cell model to study the molecular mechanisms of insulin/IGF-I-mediated differentiation processes. In these cells insulin and IGF-I, acting independently, up-regulate the expression of adipogenic-related genes (fatty acid synthase, glycerol-3-phosphate dehydrogenase, malic enzyme, glucose-6-phosphate dehydrogenase) in a dose and time-dependent manner at the transcriptional level, resulting in an increase of cytosolic lipid content [20,40].

Regarding the molecular mechanisms by which insulin/IGF-I elicit the positive effects on adipogenic differentiation of fetal brown adipocytes, the IRS/PI 3-kinase signaling pathway plays a major role. Effective inhibitors of PI 3-kinase may help to define the role of this enzyme and its products in cells. In this regard, the use of two unrelated chemical inhibitors of PI 3-kinase enzymatic activity has allowed us to define the specific contribution of this signaling pathway in inducing the expression of various metabolic genes in brown adipocytes [41]. Wortmannin is a cell-permeable fungal metabolite that acts as a potent, selective, cell-permeable, and irreversible inhibitor of PI 3-kinase (IC_{50}=5 nM) [42]. It blocks the catalytic activity of the enzyme by interfering with p85-p110 interaction without affecting upstream signaling events. When tested at μM concentrations, wortmannin did not interfere with activities of other protein kinases such as PKC, cyclic-GMP-dependent protein kinase, calmodulin-dependent protein kinase II, cyclic-AMP-dependent protein kinase of PDGF receptor tyrosine kinase. However, wortmannin can interfere at μM concentrations with myosin light-chain kinase. 2-(4-Morpholinyl)-8-phenyl-4H-1-benzopyran-4-one (LY294002) is an unrelated cell-permeable, potent, and specific PI 3-kinase inhibitor that acts on the ATP-binding site of the enzyme (IC_{50}=1.4 μM) [43]. A significant feature of the ability of LY294002 to inhibit PI 3-kinase is the effect of single atom substitutions in the morpholine ring (Fig. **3**). The simple replacement of the 4´-oxygen with sulfur, hydroxymethyl, methylene, or nitrogen causes a dramatic decrease in the efficacy of these compounds against PI 3-kinase. In addition, LY294002 did not inhibit selected tyrosine kinases (EGF receptor, c-src kinase), serine/threonine kinases (PKC, PKA, MAPK), lipid kinases (PI-4 kinase, diacylglicerol kinase) or ATPases when tested at doses that completely abolished PI 3-kinase. Experiments performed in our laboratory have shown that the expression of several adipogenic genes induced by IGF-I or insulin was completely prevented by treatment of primary brown adipocytes with either wortmannin or LY294002. As a result, the cytosolic lipid content was severely reduced [41]. Among the PI 3-kinase downstream targets, the role of p70s6k has been studied by using rapamycin (Fig. **4**). This chemical compound is a macrolide immunosuppresant that selectively inhibits the phosphorylation and activation of the enzyme (IC_{50}=50 pM) [44]. Rapamycin partly, but not totally, inhibited IGF-I-induced adipogenic gene expression, suggesting that in addition to p70s6k other molecules such as the serine/threonine protein kinase B/Akt (PKB/Akt) or protein kinase C ζ (PKCζ), that are activated downstream of PI 3-kinase, might participate in the molecular cascade leading to the nucleus where gene expression is regulated [41].

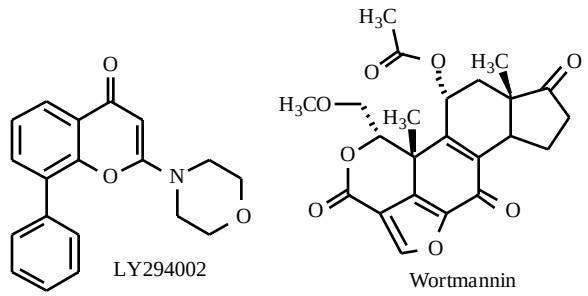

Fig. (3). Structure of LY 294002 and Wortmannin.

Another interesting theme in brown adipocyte differentiation is glucose uptake. Glucose is the main lipogenic substrate for fetal brown adipocytes [45]. Glucose transport in adipocytes is maintained mainly by the activity of the insulin-regulated glucose transporter GLUT4, although the ubiquitous GLUT1 glucose transporter is often expressed at appreciable levels [46]. Activation of PI 3-kinase mediates GLUT4 redistribution from an intracellular compartment to the plasma membrane upon insulin stimulation [47]. In brown adipose tissue, GLUT4 mRNA increased greatly at day 22 of fetal development concomitantly with the onset of adipogenic differentiation [14,48]. In primary cultures of brown adipocytes, insulin and IGF-I transactivated the GLUT4 promoter and up-regulated the expression of the GLUT4 gene, producing a time-dependent mRNA accumulation and significantly increasing the amount of protein located in total membrane fraction. Consequently, glucose uptake was augmented [49]. To address the underlying molecular mechanisms involved in insulin/IGF-I effects on GLUT4 gene expression and glucose uptake, chemical inhibitors have been extensively used. Inhibition of PI 3-kinase with either wortmannin or LY294002 down-regulated insulin-induced GLUT4 mRNA accumulation or GLUT4 protein content as well as precluded the transactivation of the GLUT4 promoter. Similar results have been obtained by transient transfection of primary brown adipocytes with constructs encoding dominant negative p85 (Δp85). However, rapamycin has no effect on either insulin-induced up-regulation of GLUT4 mRNA or protein, or on transactivation of the GLUT4 promoter, indicating that in fetal brown adipocytes p70s6k activity is not involved in the insulin signaling leading to GLUT4 expression and glucose uptake [49]. Conversely, the use of 1-(5-Chloronaphthalene-1-sulfonyl) homopiperazine (ML-9) compound (Fig. **5**) as an inhibitor of PKB/Akt enzymatic activity (IC_{50}=10-50 μM) [50] has revealed that activation of PKB/Akt is necessary for the acute (GLUT4 translocation)

and chronic (GLUT4 gene expression) insulin effects that lead to glucose uptake [51]. Although it has been reported that ML-9 is also an inhibitor of other serine/threonine kinases like PKA (IC_{50}=20 μM) and p90S6 kinase (IC_{50}=50 μM), neither of these kinases are involved in the regulation of the acute metabolic effects of insulin where insulin receptor tyrosine kinase and activation of PI 3-kinase/PKB activity play a critical role [50].

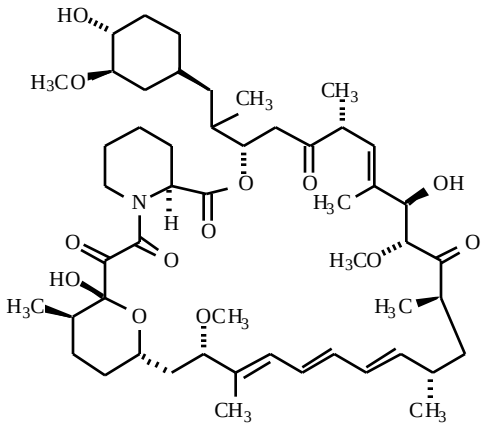

Fig. (4). Structure of Rapamycin.

Recently, the chemical compound okadaic acid has been useful to investigate the role of PKCζ (another PI 3-kinase effector) on insulin-induced glucose uptake. Okadaic acid is an ionophore-like polyether derivate of a C_{38} fatty acid compound that has tumor promoting properties (Fig. **6**) [52]. It is a potent inhibitor of protein serine/threonine phosphatase 1 (IC_{50}=10-15 nM) and protein serine/threonine phosphatase 2A (IC_{50}=0.1 nM) without any inhibitory effect on the activity of tyrosine phosphatases. Primary brown adipocytes pretreated with this compound before insulin stimulation showed a marked increase in IRS-1/2 serine phosphorylation with a concomitant decrease in tyrosine phosphorylation of these molecules and a partial inhibition of IRS-1- and IRS-2-associated PI 3-kinase activity [53]. Downstream PI 3-kinase, okadaic acid blunted insulin-induced PKCζ activity, but not PKB/Akt or p70s6k phosphorylation. The metabolic consequence of PKCζ inhibition is a blockade of insulin-induced glucose uptake. These results together with those reported by Hernandez and co-workers [51] indicate that PKB/Akt and PKCζ activation seems to be an absolute requirement for insulin-induced glucose uptake in fetal brown adipocytes.

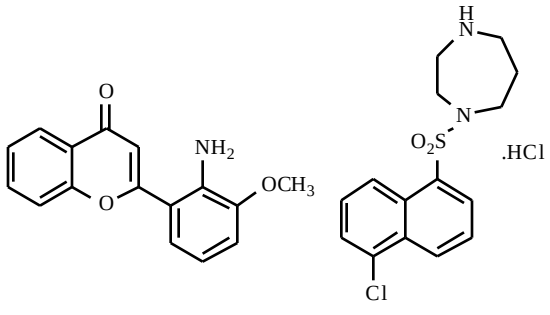

Fig. (5). Structure of PD 98059 and ML-9.

In contrast to the positive role of PI 3-kinase signaling, insulin/IGF-I activation of MAPK plays a negative role in the adipocytic differentiation of fetal brown adipocytes. The chemical compound 2´-amino-3´-methoxyflavone (PD98059) is a selective and cell permeable inhibitor of MEK (IC_{50}=2 μM) that acts by inhibiting the activation of MAPK (Fig. **5**). This compound was identified by screening a compound library with a cascade assay that measured phosphorylation of MBP (myelin basic protein) in the presence of GST-MEK1 and GST-MAPK fusion proteins [54]. Interestingly, addition of PD098059 to the assay after GST-MAPK was activated by incubation with GST-MEK1 did not result in attenuated MBP phosphorylation, indicating that PD098059 blocks the activity of MEK and not that of MAPK. In addition, PD098059 did not inhibit a number of other kinase activities both *in vitro* and in cultured cells. These include Raf kinase, PKC, cyclic AMP-dependent kinase, v-src kinase, PDGF receptor tyrosine kinase and PI 3-kinase. When brown adipocytes were cultured with PD98059 together with insulin or IGF-I an enhanced FAS mRNA expression was observed [55]. However, Fasshauer and co-workers [56] have not found an improvement in the differentiation capacity of brown preadipocytes upon inhibition of MAPK.

In addition to adipogenesis, insulin and IGF-I are novel thermogenic factors through the ability to increase UCP-1 gene transcription rate and also UCP-1 mRNA accumulation [19,21]. Moreover, insulin/IGF-I transactivates the UCP-1-chloramphenicol acetyl transferase (CAT) fusion gene in a tissue-specific manner. Inhibition of PI 3-kinase with wortmannin or LY294002, completely blocked insulin/IGF-I-induced transactivation of the UCP-1 promoter and UCP-1 mRNA accumulation [21,41]. However, p70s6k inhibition by rapamycin did not preclude the transactivation of the UCP-1 promoter induced by insulin/IGF-I [21] but partly inhibited IGF-I-induced UCP-1 mRNA expression [41]. In addition, Ras proteins seem to be involved in insulin/ IGF-I-induced UCP-1 expression [57,58]. In fact, transient transfection of primary brown adipocytes with a dominant-negative form of p21 Ras completely abolished insulin-induced UCP-1-CAT transactivation [58]. All together these results strongly suggest that IGF-I and insulin induce thermogenic differentiation of brown adipocytes by a PI 3-kinase and p21 Ras dependent manner. Whether activation of MAPK inhibits this process remains controversial.

METABOLIC CONSEQUENCES OF INSULIN RESISTANCE IN FETAL BROWN ADIPOCYTES

Insulin resistance in peripheral tissues is a characteristic clinical feature of a number of disease states including Type 2 diabetes. Several states of insulin resistance are related to receptor and postreceptor defects, such as naturally occurring mutations in the primary sequence of the insulin receptor that results in decreased insulin binding [59], a decrease in the number of insulin receptor molecules expressed on the plasma membranes of the target cells [60,61], or alterations in insulin postreceptor signaling. During the last years our laboratory has been investigating the molecular mechanisms of insulin resistance in brown adipocytes due to alterations in the early steps of the insulin signaling cascade. Transforming growth factor α (TNFα) is a potent cytokine

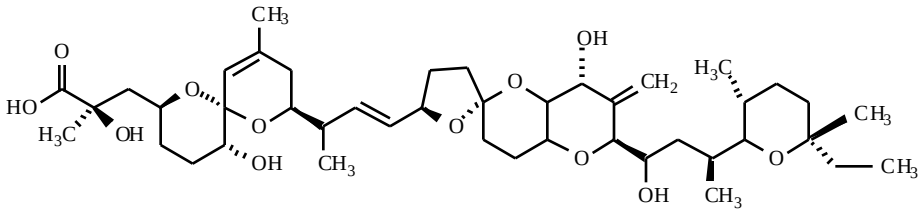

Fig. (6). Structure of Okadaic Acid.

that acts as a negative regulator of BAT differentiation. The first reported action of TNFα in brown adipocytes was the induction of insulin resistance [62]. Thus, pretreatment of rat fetal brown adipocytes in primary culture with TNFα inhibited insulin-induced expression of several adipogenic differentiation mRNAs markers such as FAS and G3PD. The antiadipogenic effect of TNFα was accompanied by a down-regulation of the mRNAs for the transcription factors C/EBPα and β. Moreover, TNFα caused an insulin resistance on the insulin-induced glucose uptake in brown adipocytes. At the molecular level, the negative actions of TNFα on brown adipocytes seemed to be due to the hypophosphorylation of the insulin receptor in response to insulin as well as the hypophosphorylation of IRS-2, resulting in an impairment of IRS-2-associated PI 3-kinase activity [62]. A step further, TNFα mediates the production of ceramide which triggers PKB/Akt dephosphorylation through the activation of a protein phosphatase [63]. However, recent data indicate that TNFα is also a direct inhibitor of adipogenic genes and UCP-1 expression acting through mechanisms mediated by TNFα receptors, but unrelated with the induction of insulin resistance [64,65].

Insulin resistance as characterized by reduced phosphorylation and activation of the insulin receptor occurs by H-ras transformation. Immortalized rat fetal brown adipocytes expressing constitutively active p21ras protein (H-ras^{lys12}) maintain the brown adipocyte phenotype showing an impaired insulin receptor and IRS-1 tyrosine phosphorylation and IRS-1-associated PI 3-kinase activity upon treatment with physiological doses of insulin [66]. Wheat-germ lectin-purified receptors from H-ras^{lys12}-transformed brown adipocytes showed a marked phosphorylation in the basal state, which was suppressed by serine/threonine phosphatase pretreatment. Moreover, alkaline phosphatase pretreatment restored the tyrosine phosphorylation and the kinase activity of the receptor in response to insulin. Consequently, these results suggest a possible feedback mechanism causing an impairment of the insulin receptor by enhancing its serine/threonine phosphorylation, which in turn decreases tyrosine phosphorylation.

ROLE OF IRS PROTEINS IN BROWN ADIPOCYTES

IRS proteins are key mediators of insulin signaling. Alterations in the expression or tyrosine phosphorylation of IRS proteins which interfere with the activation of downstream insulin signaling have been noted in adipocytes *in vitro* or in animal models of insulin resistance. For instance, IRS-1 has recently been described as an essential signaling molecule, acting through PI 3-kinase, in the differentiation of brown adipocytes *in vitro* based on protocols developed in mouse fibroblasts [67] and in brown fat preadipocytes [56]. Moreover, IRS-1 seems to be the main molecule driving insulin signaling in human white adipocytes since it is reduced in subjects with Type 2 diabetes [68]. In order to demonstrate whether IRS-1 mediates the insulin effect on brown adipocyte differentiation, immortalized brown adipocytes derived from the fetuses of IRS-1-deficient mice, as well as from the wild-type controls have been generated. It is noteworthy that these cells maintain the adipogenic- and thermogenic- phenotype of brown adipocytes when grown in culture [69,70]. Regarding signal transduction pathways, IRS-1-deficient brown adipocytes show a significant increase of IRS-2-associated PI 3-kinase activity as compared to the wild-type. Nevertheless, the overall PI 3-kinase activity (associated with anti-phosphotyrosine) was decreased by 30% in IRS-1-deficient cells. Moreover, the loss of insulin-induced PI 3-kinase activity in IRS-1$^{-/-}$ brown adipocytes was accompanied by a decrease in the phosphorylation of PKB/Akt, without changes in the phospho-70s6k content. These results suggest an interesting divergence in signaling pathway downstream of PI 3-kinase. While the lack of IRS-1 is not compensated by IRS-2 in the activation of Akt, IRS-2 may substitute in activating p70s6k. However, the existence of a PI 3-kinase-independent mechanism which activates Akt as recently suggested by Somwar and co-workers [71] can not be excluded. Finally, the lack of IRS-1 diminished the cytosolic lipid content in serum-starved homozygous and heterozygous IRS-1-deficient brown adipocytes, as compared to wild-type cells. In addition, no enhancement of the cytosolic lipid content in response to insulin stimulation was observed in IRS-1$^{-/-}$ cells, even though IRS-2 protein was increased at that time [69]. Moreover, insulin induced a significant increase in FAS mRNA in wild-type cells, but not in IRS-1$^{-/-}$ cells. These results provide consistent evidence indicating that lack of IRS-1 leads to insulin resistance at the level of adipogenic gene expression and lipid synthesis in response to insulin. As summarized in (Fig. **7**), these data clearly indicate that IRS-1/PI 3-kinase/Akt activation, but not IRS-2/PI 3-kinase activation, is required for insulin stimulation of lipid synthesis in brown adipocytes, in a p70s6k-independent manner.

In addition to its essential role in adipogenesis, IRS-1 is a key molecule in mediating insulin-induced thermogenic gene expression in fetal brown adipocytes. Insulin fails to induce UCP-1 expression and to transactivate the UCP-1 promoter in IRS-1-deficient fetal brown adipocytes due to the lack of IRS-1-associated/Akt signaling in response to insulin (Fig. **7**). Reconstitution of IRS-1-deficient brown adipocytes with the IRS-1 mutant which contains substitution of Phe for Tyr in 18 potential tyrosine

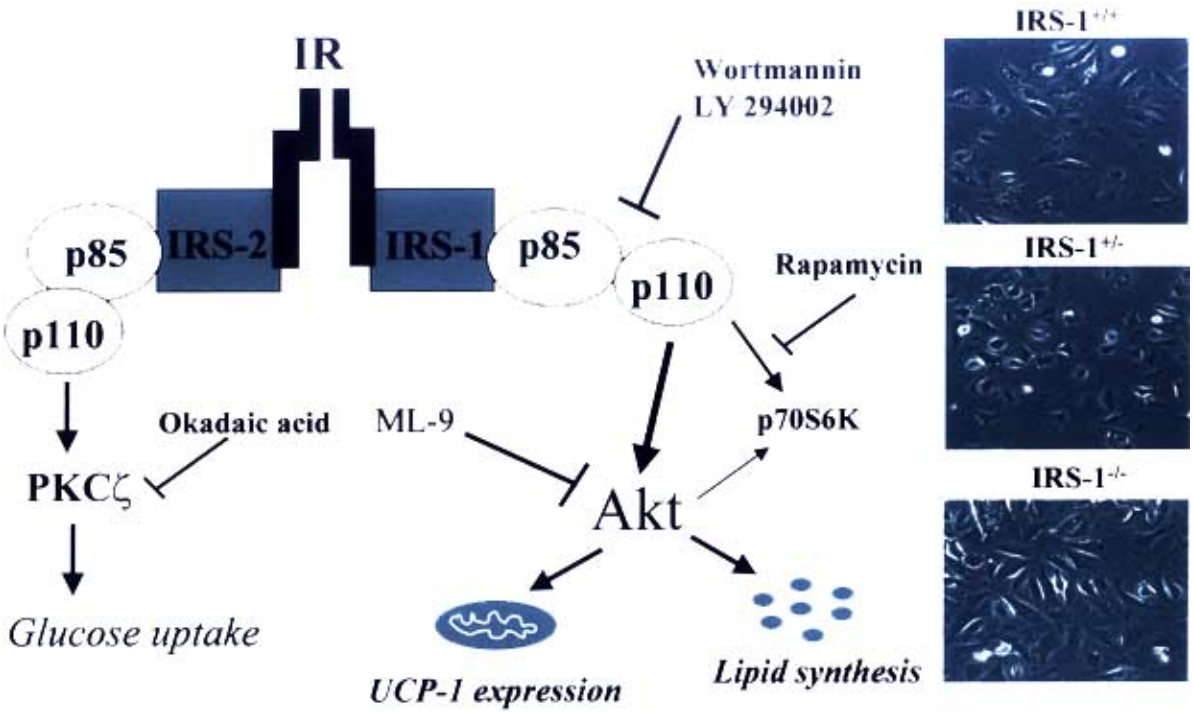

Fig. (7). Insulin Signaling Pathways Leading to Brown Adipocyte Differentiation. IRS-1/IPI 3-kinase/Akt pathway is essential for insulin-induced lipid synthesis and UCP-1 expression in brown adipocytes. IRS-2/PI 3-kinase/PKCζ mediates insulin induced GLUT4 translocation and glucose uptake.

phosphorylation sites except tyrosines at positions 608, 628 and 658 (p85 binding sites), results in a recovery of insulin-mediated IRS-1/PI3-kinase/Akt activation, transactivation of the UCP1-promoter and UCP-1 expression [72]. Interestingly, very recent findings reveal that IRS-1 is unable to mediate neither insulin-induced UCP-1 expression nor adipogenic gene expression in brown adipocytes lacking IGF-I receptors, strengthening the essential role of IGF-IR during the development of BAT [73,74].

IRS-2 is an alternative insulin receptor substrate which connects the insulin receptor with the p85 regulatory subunit of PI 3-kinase in brown adipocytes [75]. In these cells, IRS-2 seems to be crucial in mediating insulin-induced glucose uptake since insulin-stimulated glucose uptake was impaired in immortalized brown adipocytes derived from IRS-2-deficient mice [76]. However, it has been recently demonstrated that either exercise or insulin-induced glucose transport in the skeletal muscle of IRS-2 knockout mice is not altered [77], illustrating the tissue-specificity of the insulin action. Additionally, in IRS-2-deficient brown adipocytes insulin-induced effect on GLUT4 translocation to the plasma membrane was impaired [76]. Regarding the underlying molecular mechanism of insulin resistance in inducing glucose uptake, recent data indicate that PI 3-kinase/PKCζ, but not PI 3-kinase/Akt signaling pathway, is inhibited in IRS-2-deficient brown adipocytes upon insulin stimulation. Furthermore, wild-type cells expressing a kinase inactive (KI) mutant of PKCζ lack insulin responsiveness in induce GLUT4 translocation and glucose uptake [78].

Thiazolidinediones (TZD) are a new class of antidiabetic agents that act by improving insulin sensitivity in various animal models of diabetes as well as in humans. TZD induce their diverse effects by binding to and activating the peroxisome proliferator- activated receptor (PPAR)γ [79]. It is currently unknown how TZD improve insulin sensitivity since known PPARγ-regulated genes are mainly involved in adipocyte differentiation, lipid storage and metabolism [80]. One current hypothesis is that these drugs induce the formation of new, small, and insulin sensitive fat cells reducing the production of TNFα, a cytokine which impairs insulin signaling and has been implicated in obesity-induced insulin resistancee [81]. In addition, increased GLUT4 expression has been reported in adipocytes treated with TZD [82]. In brown adipocytes, TZD increase UCP-1 expression [83,84]. This is an important feature since UCP-1 expression in white fat induced by pharmacological treatment may reduce adiposity and improve insulin sensitivity. Regarding TZD action on insulin signaling, IRS-2 protein expression increased after TZD treatment in murine 3T3-L1 and human adipocytes [85]. Since reduced IRS-2 expression or impaired IRS-2 signaling appears to play a profound role in the development of diabetes [86,87], these data suggest that the antidiabetic effect of TZD may be mediated at least in part through this pathway. It will be of great interest to determine whether TZD can influence beta cell growth and/or apoptosis, since IRS-2 disruption has a stunning effect on pancreatic beta cell development.

ROLE OF BAT IN THE CONTROL OF GLUCOSE HOMEOSTASIS

In rodents, glucose is an important fuel for BAT *in vivo*. Under physiological insulin stimulation, BAT has an extremely high rate of glucose metabolism (10% of the total

glucose turnover rate in the rat) [88,89], and a number of metabolic genes are induced. Among them, insulin plays an important role in the regulation of UCP-1 expression *in vivo* [90]. Indeed, the maintenance of normal concentrations of UCP-1 in BAT has been shown to require normal plasma insulin levels [91]. However, the fact that hyperglycaemia partially prevents the rapid decrease in the concentration of UCP-1 and its mRNA observed in streptozotocin-induced diabetes suggests the possibility of a glucose-dependent mechanism in the regulation of UCP-1 expression [90]. Conversely, insulin resistance in BAT appears in different physiological situations. In pregnant rats, the increase in glucose utilization linked to an increment in plasma insulin is significantly decreased indicating that BAT seems to take part in the overall insulin resistance which characterizes late pregnancy [88]. During lactation, the effect of insulin on total glucose metabolism is decreased by 50% in rat brown adipocytes. This alteration reflects a specific lack of insulin action on glucose oxidation and lipogenesis in BAT and is necessary for preserving substrates for the synthesis of milk components in the mammary gland [88,92]. In addition, decreased responsiveness to insulin in BAT has been reported in obese Zucker rats [88]. All together these data identify the role of BAT in the overall control of glucose homeostasis.

The ability to modify genes in a mouse has allowed us and others to create, in a general and in a tissue-specific fashion, animal models to assess the role of insulin receptor-mediated signaling [93]. The peripheral insulin resistance associated with Type 2 diabetes has been demonstrated in muscle, liver and WAT. However, little is known about the potential role of insulin action or insulin resistance in BAT in the development of this disease. Previous work reported by Angel *et al*. [94] demonstrated that GLUT4 mRNA and protein levels are decreased in WAT and BAT of Type 2 diabetic rats. Accordingly, in order to assess whether insulin signaling in BAT participates in the control of glucose homeostasis, brown adipose tissue-specific IR knockout mice (BATIRKO) has been generated [95]. Analysis of BATIRKO mice has revealed two potentially important aspects of insulin resistance in this tissue with respect to whole body glucose homeostasis. First, insulin plays a role in the development or maintenance of BAT, as previously suggested in the *in vivo* and *in vitro* studies. Thus, these animals display a significant brown fat atrophy which is age-dependent. This phenotype is further reflected by a severe reduction in the expression of the lipogenic genes seen in the remnant interscapular BAT. The second phenotype observed in BATIRKO mice is an altered regulation of glucose homeostasis. These animals developed a diabetic phenotype without peripheral insulin resistance but with moderate decrease in beta cell mass and a marked insulin-secretion defect in response to glucose *in vivo* and in isolated islets. Consequently, BATIRKO is an interesting animal model of diabetes which suggests that the dysregulation of an adipoinsular axis due to the lack of insulin receptor in BAT alters glucose homeostasis.

FUTURE PERSPECTIVES

Insulin resistance is a common component of the metabolic syndrome, together with several physiopathological features such as hypertension, obesity, hyperlipemia and Type 2 diabetes. The combination of two or three of the above factors confers a serious risk of cardiovascular damage, since insulin resistance is involved in vascular disease also at pre-diabetic stages. The treatment of insulin resistance and Type 2 diabetes has been restricted to the administration of exogenous insulin and sulfonylureas, which promote the release of endogenous insulin. Although they are effective in reducing serum glucose levels in diabetic patients, neither of these agents addressed the underlying insulin resistance of the core of the metabolic syndrome. More recently, metformin, an inhibitor of hepatic glucose production, has been used in the treatment of Type 2 diabetes because ameliorates hepatic insulin resistance. However, the use of metformin in patients with significant renal, hepatic, or cardiac disease can lead to lactic acidosis. The recent introduction of the TZD insulin-sensitizing drugs has allowed us to move from the bench to the bedside to test speculations about the pharmacological treatment of insulin resistance. TZD reduce insulin resistance and improve glucose tolerance in diabetic rodents and humans by a complex mechanism not yet completely understood. However, several limitations have now emerged. Firstly, TZD increase adiposity and trigger an undesirable secondary insulin resistance in WAT. Secondly, TZD induce the expression of the scavenger receptor CD36 in macrophages and monocytes that mediates the uptake of oxidized LDL involved in foam cell development during atherogenesis. Furthermore, TZD also induce CD36 in peripheral tissues such as WAT that may contribute to the removal of oxidized LDL from the circulation. More importantly, atherosclerosis is a complex disease with multiple inputs, including physiological parameters such as blood pressure and lipid levels, as well as intrinsic components of the vessels such as vascular smooth muscle migration and the production of thrombotic proteins by vascular endothelium. In this regard, the use of TZD reduce blood pressure in several mammalian models and also smooth muscle proliferation *in vitro* and *in vivo.* Thus, TZD reveal evidence of both pro- and anti-atherogenic effects. The hope is that future generations of PPARγ ligands will explore, in combination with inhibitors of HMGCoa reductase, their antiatherogenic properties to the fullest.

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ABBREVIATIONS

NIDDM	=	Type 2 diabetes mellitus
BAT	=	Brown adipose tissue
WAT	=	White adipose tissue
FFA	=	Free fatty acids
ATP	=	Adenosin triphosphate
GDP	=	Guanosin diphosphate
GTP	=	Guanosin triphosphate

NA = Noradrenaline
IGF-I = Insulin-like growth factor-I
IRS = Insulin receptor substrate
SH2 = Src homology 2
PI 3-kinase = Phosphatidylinositol 3-kinase
Grb-2 = Growth factor receptor binding protein-2
PKC = Protein kinase C
PKB = Protein kinase B
Akt = Protein kinase B
P70s6k = P70s6 kinase
mTOR = Mammalian target of rapamycin
MEK = Mitogen-activated protein kinase kinase
MAPK = Mitogen-activated protein kinase
RSK = Ribosomal S6 kinase
FAS = Fatty acid synthase
G3PD = Glycerol-3-phosphate dehydrogenase
UCP-1 = Uncoupling protein-1
TNFα = Transforming growth factor alpha
CAT = Cloramphenicol acetyl transferase
TZD = Thiazolidinediones
LDL = Low-density lipoprotein

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