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Review

Molecular mechanism of insulin resistance

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Free fatty acids are known to play a key role in promoting loss of insulin sensitivity, thereby causing insulin resistance and type 2 diabetes. However, the underlying mechanism involved is still unclear. In searching for the cause of the mechanism, it has been found that palmitate inhibits insulin receptor (IR) gene expression, leading to a reduced amount of IR protein in insulin target cells. PDK1-independent phosphorylation of PKCε causes this reduction in insulin receptor gene expression. One of the pathways through which fatty acid can induce insulin resistance in insulin target cells is suggested by these studies. We provide an overview of this important area, emphasizing the current status.

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1. Introduction

Diabetes is of two types. Type 1 or insulin-dependent diabetes mellitus (IDDM) is basically due to autoimmune-mediated destruction of the pancreatic β islets resulting in insulin deficiency. Patients with type 1 diabetes usually have to take exogenous insulin for survival and to prevent the development of ketoacidosis. Type 2 diabetes or non-insulin dependent diabetes mellitus (NIDDM) is characterized by insulin resistance and is usually associated with abnormal insulin secretion. Globally, more than 90–95% of cases of diabetes are of this kind, while type 1 accounts for only 3–5% (Zimmet 1999; Zimmet *et al* 2001). The growing incidence of insulin resistance and type 2 diabetes is seriously threatening human health globally. At the dawn of the new millennium, when WHO announced that the world was facing an epidemic of type 2 diabetes, the major question that was confronting us is what really causes this insidious disease.

This review deals with the mechanisms related to type 2 diabetes. Numerous reports demonstrate that oversupply of lipids raises the circulating level of free fatty acid (FFA) and contributes to the development of type 2 diabetes (Boden 1997; Mc Garry 2001. The excess FFA is ultimately stored in non-adipose depots leading to increased intramyocellular lipids, which cause insulin resistance (Boden *et al* 2001; Kuhlmann 2003). Initiation of insulin resistance or loss of insulin activity is the primary expression of this disease.

Although it is now fairly well established that FFAs are the major players effecting insulin resistance, the underlying mechanism by which this happens is still unclear. Inhibition of glucose transport by FFA has been found to be linked to insulin-mediated signals. Binding of insulin to the heterotetrameric membrane receptor results in insulin receptor substrate-1 (IRS-1) phosphorylation and IRS-1-associated phosphatidylinositol 3 phosphate kinase (PI3 kinase) activation (Dresner *et al* 1999; Yu *et al* 2002; Chavez *et al* 2003). This affects downstream effectors such as Akt/

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Abbreviations used: IDDM, insulin-dependent diabetes mellitus; NIDDM, non-insulin dependent diabetes mellitus; IR, insulin receptor; FFA, free fatty acid; IRS, insulin receptor substrate; PI3 kinase, phosphatidylinositol 3 phosphate kinase; PIP_2, phosphatidylinositol 4,5 bisphosphate, DAG; diacylglycerol; PDK1, phosphoinositide-dependent kinase-1.

PKB which activates the glucose transporter Glut4; Glut4 is then translocated to the membrane and imports glucose into the cell (Kohn *et al* 1996; Tanti *et al* 1997; Hill *et al* 1999). This suggests that the impairment of insulin activity leading to insulin resistance is linked to insulin signalling defects. Insulin signalling molecules involved in metabolic and mitogenic action may also play a role in cellular insulin resistance (Zick 2001; White 2002; Greene *et al* 2004). A few recent reports indicate that some PKC isoforms may have a regulatory effect on insulin signalling. The expression levels and activity of a few PKC isoforms are found to be associated with insulin resistance. Lipid infusion in rats and humans impairs insulin-stimulated glucose disposal in muscle concomitantly with the activation of certain PKC isoforms (Griffin *et al* 1999; Yu *et al* 2002; Boden and Shulman 2002). We have recently demonstrated that FFA-induced insulin resistance is linked to insulin receptor gene downregulation, in which a PKC isoform plays a significant role (Dey *et al* 2005). Although much progress has been made in understanding the mechanism of FFA-induced insulin resistance and type 2 diabetes, several lacunae exist in this important field. In this review we briefly describe these lacunae along with the current status of the problem.

2. Insulin receptor and signalling

The action of insulin is initiated through its binding with the target cell surface receptor that activates tyrosine kinase, a constituent of the receptor molecule. The insulin receptor (IR) is a heterotetramer consisting of two α subunits and two β subunits that are linked by disulphide bonds into an $\alpha_1\alpha_2$ and $\beta_1\beta_2$ heterotetrameric complex. Insulin binds to the extracellular α subunit and transduces signals across the plasma membrane, which activates the intracellular tyrosine kinase C terminal domain of the β subunit. Binding of insulin to IR effects a series of intramolecular transphosphorylation reactions, where one β subunit phosphorylates its adjacent partner on a specific tyrosine residue (Pessin and Saltiel 2000). Although IRs are present on the surface of virtually all cells, their expression in classical insulin target tissues, i.e. muscle, liver and fat, is extremely high (Brunetti *et al* 2001). However, there is very little information on the regulatory mechanism that controls the IR at the level of gene expression. Autophosphorylation of the IR tyrosine residue stimulates the catalytic activity of receptor tyrosine kinase which recruits IRS proteins (IRS-1 and IRS-2) (Rosen 1987; Pessin and Saltiel 2000). These, in turn, augment the activity of the effector enzyme (Yenush *et al* 1998; White

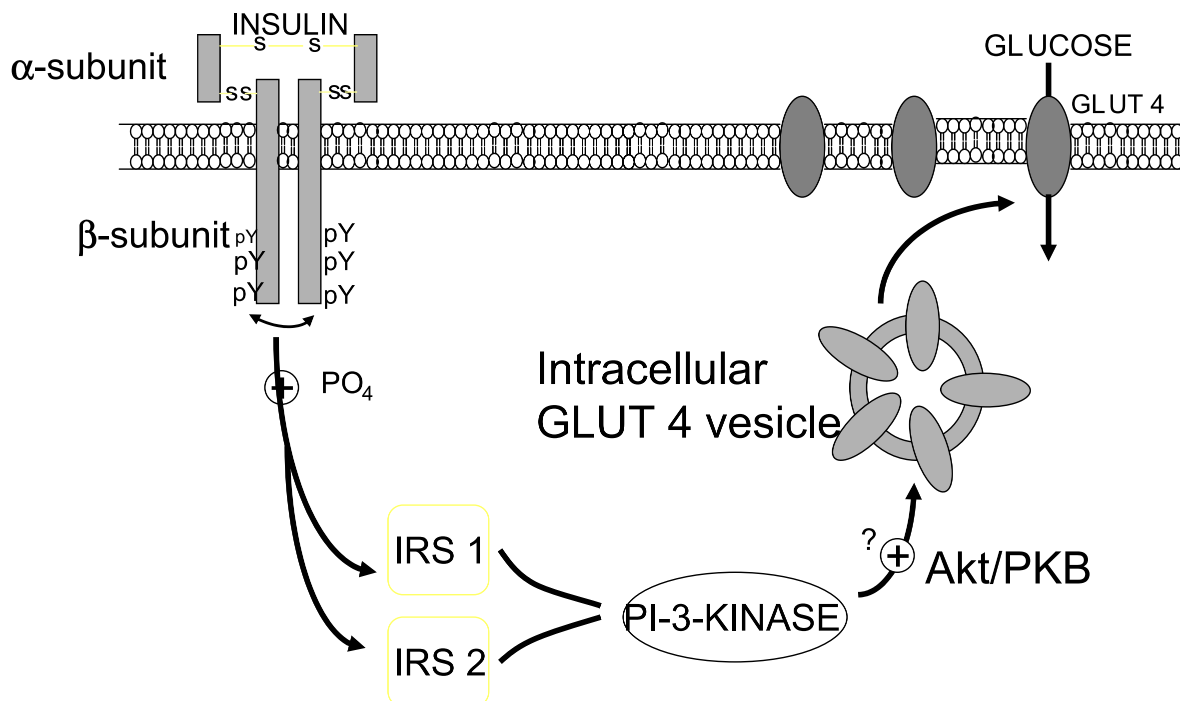

Figure 1. Insulin signalling pathway showing binding of insulin with the IR leading to activation of Glut4 which imports glucose into the cell. Activated insulin receptor tyrosine kinase phosphorylates IRS and PI3 kinase. PI3 kinase produces PI4,5 P_2 and PI3,4,5 P_3. These serve as docking sites for two ser/thr kinase PH domains, i.e. PDK1 and Akt/PKB. Binding of Akt/PKB to PIP3 activates Glut4. Activated Glut4 is translocated to the plasma membrane to transport glucose into the cell.

1998). PI3 kinase is a target of the IRS proteins (IRS-1 and IRS-2) which phosphorylates specific phosphoinositides to form phosphatidylinositol 4,5 bisphosphate (PIP_2) to phosphatidylinositol 3,4,5 triphosphate; in turn, this activates ser/thr kinase, i.e. phosphoinositide-dependent kinase-1 (PDK1) (LeGood *et al* 1998). Activated PDK1 phosphorylates or activates ser/thr kinase Akt/PKB. Akt contains a PH domain that also interacts directly with PIP3 (Saltiel and Kahn 2001). Akt plays an important role by linking Glut4, the insulin-dependent glucose transporter protein, to the insulin signalling pathway. It activates Glut4 which moves to the cell surface to transport glucose into the cell (Pessin *et al* 1999; Kupriyonova and Kendror 1999; Martin *et al* 2000). Figure 1 briefly describes the insulin signalling pathway operative in the insulin target cell.

3. FFA-induced impairment of insulin signalling

Insulin has 3 major target tissues—skeletal muscle, adipose tissue and liver. Not only is the IR overexpressed in the cells of these tissues but these are also the three places where glucose is deposited and stored; no other cells can store glucose. In normal subjects the blood glucose level normally ranges between 90 and 120 mg/dl; after a meal this may go up to 250–300 mg/dl. The fasting insulin level remains at 15 μU/ml and after the intake of food it may go up to 40 μU/ml. This increase in insulin secretion is responsible for the post-prandial decrease in glucose which reaches the normal level after about 2 hours. Removal of this excess glucose by insulin occurs due to glucose uptake and storage in insulin target cells such as skeletal muscle cells, hepatocytes and adipocytes. About 75% of this glucose is stored in skeletal muscle cells; therefore, it is the major target cell of insulin, while the rest is stored in the liver and adipocytes. When an increase in insulin after a meal cannot take care of this process, the higher glucose in the circulation remains for a longer period, as occurs in type 2 diabetes. Patients suffering from insulin resistance and type 2 diabetes frequently display signs of abnormal lipid metabolism, increased circulatory concentration and elevated deposition of lipids in the skeletal muscle (Boden 1997; Mc Garry 2001). Increase in plasma lipid levels impairs insulin activity, increase in plasma FFA reduces insulin-stimulated glucose uptake, whereas a decrease in lipid content improves insulin activity in the skeletal muscle cells, adipocytes and liver (Moller 2001).

One of the sites that appears to be involved in fatty acid-induced insulin resistance is the IR itself. There are different ways by which fatty acids could impose insulin resistance. These are changes in IR expression, binding of ligands, the phosphorylation state of its kinase domain and the activity of receptor tyrosine kinases could account for insulin resistance. It could be a functional defect or may be related to genetic defects in the IR that influences its expression, binding to ligands and tyrosine kinase activity (Pessin and Saltiel 2000). Interesting results are obtained by studying IRs in knockout mice. Homozygous IR-null mice die shortly after birth owing to extreme insulin resistance (Accili *et al* 1996; Joshi *et al* 1996). IRS-1 is an important protein and homozygous knockout mice lacking a single allele of this gene lack any significant phenotype, whereas homozygous disruption of the *IRS-1* gene results in a mild form of insulin resistance (Araki *et al* 1994). IRS-1 homozygous null mice ($IRS\text{-}1^{-/-}$) do not show a clear diabetic phenotypic expression, presumably because of pancreatic β cell compensation. However, mice that are doubly heterozygous ($IR^{-/+}$, $IRS^{-/+}$) develop both insulin resistance and diabetes (Bruning *et al* 1997). Homozygous disruption of the *IRS-2* gene, on the other hand, causes impairment of insulin secretion along with peripheral insulin resistance and diabetes (Withers *et al* 1998). The insulin resistance observed in IRS-2 knockout animals probably reflects secondary events due to alteration in β cell function as IRS-2 appears to be unnecessary for insulin or exercise-stimulated glucose transport (Higaki *et al* 1999). This observation is consistent with the results of studies in β cell-specific IR knockout mice, which develop peripheral insulin resistance and diabetes, most probably due to the changes in the pattern of insulin secretion (Kulkarni *et al* 1999).

FFA also affects downstream insulin signalling molecules. It inhibits insulin activation of IRS-1-associated PI3K activity in muscle. Reduction of insulin-stimulated IRS-1 tyrosine phosphorylation and IRS-1-associated PI3K activity by FFA has been shown to be associated with an increase in IRS-1 serine phosphorylation. This in turn decreases IRS-1 tyrosine phosphorylation, impairing down stream effectors (Yu *et al* 2002). FFA can disrupt further downstream insulin signals. Administration of saturated fat blocks insulin activation of Akt/PKB with a concomitant increase in the amount of ceramide and diacylglyerol in cultured muscle cells (Chavez *et al* 2003). Insulin actively sequesters Glut4 at an intracellular location which increases the rate of Glut4 trafficking to the membrane (Martin *et al* 2000). Glut4 is the penultimate molecule in the signal and its translocation to the membrane by insulin is the ultimate step in signalling, as only then is glucose transported into the cell. Lipid-associated insulin resistance has also been shown to be linked to Glut4 translocation defects (Pessin *et al* 1999).

4. Mechanism of insulin resistance

From the above description it is clear that FFAs induce defects in the insulin signalling pathway. However, the underlying mechanism of FFA-induced impairment of insulin signals is still unclear. Does FFA cause a defect in the IR leading to reduced efficiency in formation of the insulin–IR complex, or does it indirectly regulate IR activity which adversely

affects downstream signalling molecules? Is the effect of FFA localized to a number of signalling points? These questions have not been answered. It has been suggested that insulin signalling molecules involved in the metabolic action of insulin also play a role in cellular signalling (Zick 2001; White 2002; Greene *et al* 2004). Some of the PKC isoforms represent such signalling molecules. PKC isoforms are classified as classical (cPKCα, βI, βII, γ), novel (nPKC δ, ε, θ, η) and atypical (aPKC ζ, λ). cPKCs are activated by Ca^{+2} and diacylglycerol (DAG), nPKCs are activated by only DAG and aPKCs respond to neither Ca^{+2} nor DAG (Newton 2003). Among all these PKC isoforms, nPKCs are said to have a modulatory role in insulin signalling. Recent reports also demonstrate a link between nPKCs and FFA-induced insulin resistance. Lipid infusion in rats and humans impaired insulin-stimulated glucose disposal into the muscle and concomitant activation of PKCθ and PKCδ (Boden and Shulman 2002; Itani *et al* 2002; Yu *et al* 2002). PKCδ has been shown to be a possible candidate for phosphorylation of the IR on serine residues, which obviously decreases tyrosine phosphorylation of the IR and affects its routing (Strack *et al* 1997). PKCδ has been shown to be regulating the state of IR phosphorylation (Kellerer 1997). Overexpression of PKCδ in cultured myotubes stimulates serine phosphorylation of the IR in response to insulin. Since tyrosine phosphorylation of IR is the requirement to initiate the insulin signal, phosphorylation of serine would reduce insulin signalling efficiency. Overexpression of PKCδ in cultured myotubes stimulates serine phosphorylation of IR in response to insulin. Serine phosphorylation of IR changes IR distribution on the cell membrane; a rich component of it is localized on the internal membrane instead of extracellular domain thus causing attenuation of insulin-induced tyrosine phosphorylation of the IR (Braiman *et al* 2001). These result in defects in the insulin signalling pathway imposing insulin resistance. Interestingly, activation of PKCδ and PKCθ occurs along with FFA-induced insulin resistance in muscle cells (Boden and Shulman 2002). Suppression of endogenous glucose production in the liver by insulin is effectively inhibited by FFA with concomitant activation of PKC. What is still not clear is the mechanism involved in PKCδ-mediated inhibition of IR activity and whether it is direct or indirect. One report demonstrated that PKCδ can directly inhibit IR kinase-stimulated IRS-1 associated tyrosine phosphorylation (Greene *et al* 2004). This has also been suggested to occur due to lipid-induced PKCθ activation in muscle (Schmitz-Peiffer *et al* 1997; Griffin *et al* 1999). We have recently shown that PKCδ phosphorylation by insulin is further augmented by an FFA in 3T3L1 adipocyte cells and this is associated with the inhibition of insulin-stimulated IR β and IRS-1 tyrosine phosphorylation (Dey *et al* 2006). The results of these studies promise some understanding of the occurrence of FFA-induced insulin resistance. However, the precise pathway by which FFA induces impairment of signalling molecules leading to insulin resistance still remains unclear.

5. FFA-induced activation of PKCε is linked to inhibition of *IR* gene expression

Clearly, the IR is one of the major targets in FFA-induced impairment of insulin activity. It is true that the molecular mechanism of insulin resistance is sufficiently complex. At the cellular level, it may occur at multiple steps of the insulin signalling pathway. Recent reports convincingly indicate that nPKCs, i.e. PKCδ, PKCθ and PKCε implement FFA-induced insulin resistance, which is closely associated with the attenuation of IR activity (Itani *et al* 2002; Boden and Shulman 2002; Greene *et al* 2004). However, a clear link between FFA-induced nPKC activation and impairment of the IR has not been demonstrated. Recent evidence has shown that PDK1 can directly phosphorylate all PKCs including nPKCs (Toker and Newton 2000). PDK1 is the key upstream kinase in the insulin signalling pathway and all PKCs and Akt activation are known to be dependent on it (Alessi *et al* 1997; Newton 2003). Phosphorylated Akt then activates Glut4, which moves to the membrane to import glucose.

The PKCε isotype has recently been shown to be related to insulin resistance. Translocation of PKCε into the membrane is necessary for its phosphorylation by PDK1. PKCε is overexpressed in the skeletal muscle of diabetic animals and humans, and its association with the plasma membrane causes its activation. This overexpression and activation of PKCε leads to the downregulation of IR copy number on the cell membrane along with a decrease in Akt activity (Ikeda *et al* 2001; Itani *et al* 2002). Although these reports are interesting as they implicate a novel pathway of insulin resistance and diabetes, the underlying mechanism involved in PKCε-mediated insulin resistance has not yet been elucidated. We have recently shown a possible relationship between FFA-induced PKCε phosphorylation and concomitant downregulation of IR. To understand the mechanism, we selected adipocytes and skeletal muscle cells from rat as well as respective cell lines. We observed that insulin stimulation of PDK1 phosphorylation is inhibited by an FFA, i.e. palmitate. PKCε phosphorylation is dependent on PDK1; FFA incubation of skeletal muscle cells and adipocytes inhibited PDK1 phosphorylation but surprisingly increased PKCε phosphorylation. Inhibition of PDK1 by FFA is reflected in Akt phosphorylation as Akt phosphorylation is also dependent on PDK1 (figure 2). These results are conflicting as FFA is expected to increase the DAG content in a cell, which would influence PKCε localization towards the cell membrane (Stahelin *et al* 2005) and PDK1 would phosphorylate PKCε since it is a

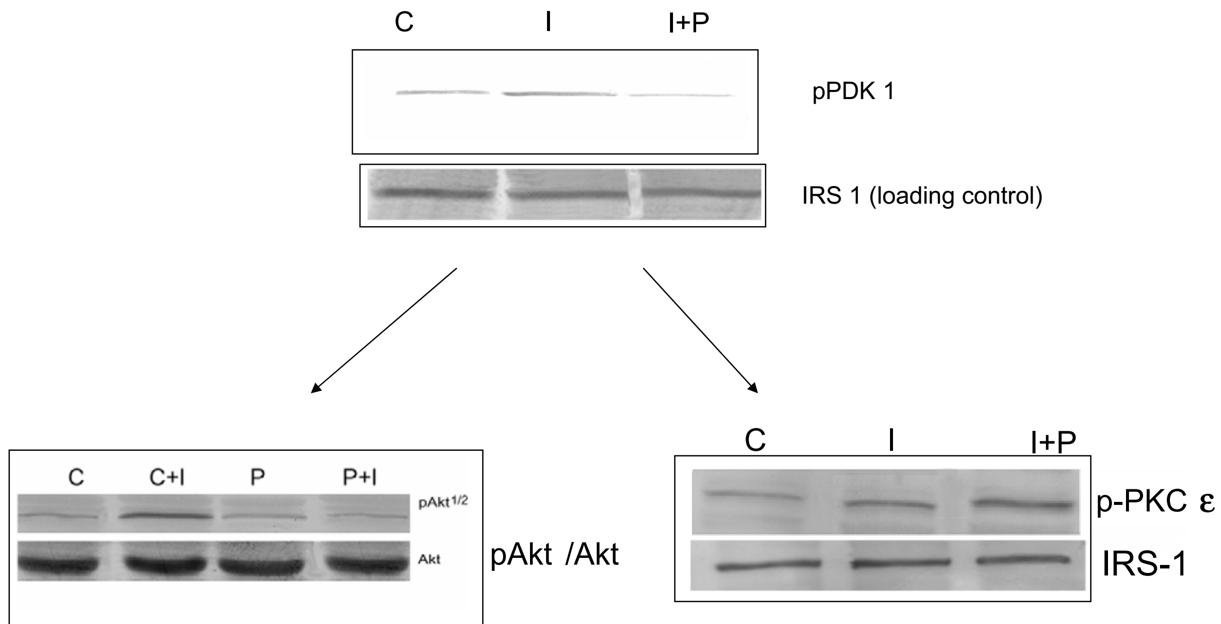

Figure 2. Effect of FFA on insulin-stimulated downstream signal, PDK1, Akt and PKCε. Skeletal muscle cells were treated without or with palmitate for 8 h and insulin was added to both control (I) and palmitate (I+P) incubated cells. Cells without any of the treatments were taken as control (C). On termination of incubation, cells were lysed by sonication and 50 μg protein from each cell lysate was resolved in 10% SDS-PAGE and then subjected to Western blot analysis with anti-pPDK1, pAkt/Akt or anti-pPKCε antibodies (1:1000). (Dey *et al* 2005).

PDK1-dependent PKC isoform. This is the known pathway of nPKC phosphorylation by PDK1; on the contrary, PKCε showed PDK1-independent phosphorylation due to FFA (Dey *et al* 2005; Dey *et al* 2006). We could not understand how PDK1-independent phosphorylation of PKCε could occur due to FFA. This remained a mystery until we came across an interesting report describing constitutive phosphorylation of PKCε by FFA in a PDK1-independent manner. This report showed that myristic acid incubation of HEPG2 cells causes myristoylation of PKCε which results in constitutive phosphorylation of PKCε at thr566/ser729 in the kinase domain required for PKCε activity. This phosphorylation was totally independent of PDK1, which the workers demonstrated by using PDK1 knockout cells. This PDK1-independent phosphorylation of PKCε could not be further increased by co-transfecting the *PDK1* gene, or decreased with kinase-inactive PDK1 transfection (Cenni *et al* 2002). In the same way, addition of palmitate to skeletal muscle cells or adipocytes may affect palmitoylation of PKCε resulting in constitutive phosphorylation of PKCε (Dey *et al* 2005; Dey *et al* 2006). We also found that activation of PKCε is inversely related to IR gene expression, palmitate decreases IR mRNA in a time-dependent manner, while inhibitors of phosphoPKCε block this decrease. Palmitate activation of PKCε occurs at 2 h in the plasma membrane. At 6 h most of the phosphorylated PKCε is associated with the nuclear fraction indicating translocation of pPKCε from cytosol to the nuclear region. This appears to be related to the inhibition of IR gene transcription. Considerable inhibition of IR gene transcription occurs at 6 h (Dey *et al* 2005). Taken together, it is clear that FFA causes PDK1-independent phosphorylation of PKCε which in turn translocates to the nucleus, and its time of entry into the nucleus coincides with inhibition of IR gene transcription. This would obviously reduce the copies of IR protein on the insulin target cell membrane. However, it is still unclear how pPKCε reduces IR gene expression on entering the nucleus.

6. Molecular mechanism of FFA-induced inhibition of IR gene transcription

The IR is usually expressed at low levels in all cell types except in classical insulin target tissues, i.e. skeletal muscle, liver and fat, where IR is expressed at a very high level (Goldfine 1987). Many workers have suspected that the IR is the major

component responsible for the loss of insulin sensitivity or insulin resistance. A few earlier reports indicate that the IR is of major importance in certain states of insulin resistance in humans, where qualitative and quantitative abnormalities of the receptor may cause defects in transmembrane signalling (Taylor 1992; Polonsky *et al* 1996; Taylor 1999; Virkamaki *et al* 1999). However, very little is known about the regulatory mechanism controlling gene expression of the IR. To understand the molecular basis of regulation of IR gene expression, the promoter region of the human IR gene has been identified and studied by several groups (Mitchell and Tjian 1989; Seino *et al* 1989; Lee *et al* 1992). The IR promoter region extends over 1800 bases upstream from the IR gene ATG codon and is extremely GC rich. It contains a series of GGGCGG repeats that are putative binding sites for the mammalian transcription factor SP1 (Mitchell and Tjian 1989). It lacks a TATA box or consensus initiator sequence and includes multiple transcription initiator sites within the first 300 bp GC-rich region. Two unique AT-rich sequences, C2 and E3, within the IR gene promoter have been identified and both these sequences are positively regulated by the architectural transcription factor HMGA1 (earlier known as HMG1-Y), a member of the high mobility group protein family (Brunetti *et al* 2001). HMGA1 interacts with the AT-rich regions in the minor groove of DNA through highly conserved DNA-binding peptide motifs termed AT hooks, and regulates transcriptional activation of many genes by modifying DNA conformation, which permits recruitment of transcriptional factor to the transcription start site (Bustin and Reeves 1996; Reeves 2001). HMGA1 protein has the ability to bind to DNA packaged in nucleosomes and regulates the expression of a number of genes through their migration from the heterochromatin to euchromatin region (Reeves *et al* 2000; Reeves 2001; Reeves and Beckerbauer 2001). It has been shown that inhibition of HMGA1 in cells that naturally express high IR levels significantly reduces cell surface expression of IR. Inversely, cells with relatively low IR content, when transfected with HMGA1, show IR overexpression and a significant increases in IR protein expression (Brunetti *et al* 2001). All these indicate that HMGA1 plays an important role in regulating transcription of the IR gene. The same group of workers has recently

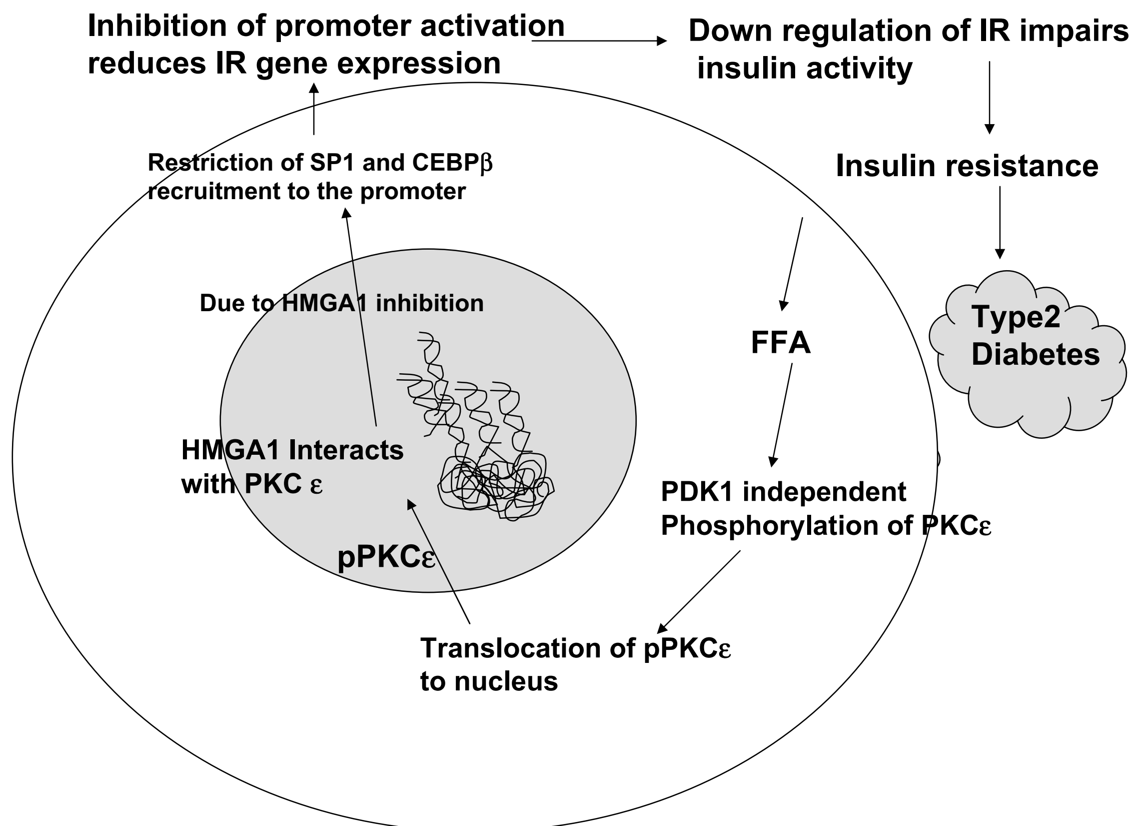

Figure 3. Schematic representation of the mechanism involved in insulin resistance. Excess of lipid mobilization to the major insulin target cells effects constitutive phosphorylation of PKCε, and that translocates to the nuclear region and reduces IR promoter activation. Inhibition in HMGA1 gene expression restricts recruitment of SP1 and CEBPβ to the IR promoter causing inhibition of promoter activation leading to downregulation of insulin receptor gene expression.

shown that HMGA1 induces transcriptional activation of the human IR gene by permitting the recruitment of SP1 and cEBPβ, the ubiquitously expressed transcription factors, to the promoter region. HMGA1 plays the role of an architectural transcription factor here as mutational interference of the HMGA1 binding site in the IR promoter abolished its binding, which adversely affected recruitment of SP1 and cEBPβ to the IR promoter, resulting in inhibition of IR promoter activation (Foti *et al* 2003).

A recent report demonstrates that a genetic flaw which reduces the intracellular expression of HMGA1 protein can adversely affect IR expression in cells and tissues from subjects with insulin resistance and type 2 diabetes (Foti *et al* 2005). There could be another aspect in the regulation of IR gene expression involving HMGA1. We have demonstrated that FFA-induced downregulation of the IR gene is associated with PDK1-independent constitutive phosphorylation of PKCε in major insulin target cells. Phospho PKCε is translocated to the nucleus which causes inhibition of IR gene expression. But how phosphorylated PKCε (pPKCε) downregulates the IR gene is unclear. Further work using PKC inhibitors showed that their administration along with FFA could block inhibition of IR gene expression (Dey *et al* 2005; Dey *et al* 2006). The question is how is pPKCε related to the regulation of IR gene expression. There is a possibility that activated PKCε phosphorylates HMGA1 which inhibits HMGA1 mobilization to the promoter region IR gene. It has been shown that phosphorylation of HMGA1 protein reduces its DNA-binding ability (Reeves and Beckerbauer 2001). Phosphorylated HMGA1 protein would preferentially interact with positively charged histones as there would be an additional negative charge, while dephosphorylated HMGA1 proteins are expected to interact with negatively charged DNA (Harrer *et al* 2004). Since HMGA1 proteins are highly mobile and this mobility is finely tuned, hyper- and hypophosphorylation of these could be the major factors in regulating the expression of the IR gene. Without the mobilization of HMGA1 to the IR promoter there is no recruitment of transcription factor to the promoter region of the IR gene and therefore no expression of the IR gene.

Although it is well established that FFA is responsible for insulin resistance as its increase in the milieu of major insulin-sensitive cells causes insulin resistance by reducing insulin sensitivity, the molecular mechanism involved is largely unknown. This review focuses on the current trends in research in this important domain and throws light on certain possibilities regarding the manner in which FFA inhibits insulin activity. The available information suggests that the IR is one of the most important sites identified so far for loss of insulin sensitivity. Figure 3 schematically represents the present status of knowledge in this field. Excess of lipid mobilization to the major insulin target cells will effect constitutive phosphorylation of PKCε, which when translocated to the nuclear region somehow reduces IR promoter activation. Inhibition of *HMGA1* gene expression in diabetic patients has recently been demonstrated, but how this is linked to FFA is unclear. However, there may be a possibility that FFA-induced pPKCε might play a role in this particular area.

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