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PI 3-KINASE AND ITS UP- AND DOWN-STREAM MODULATORS AS POTENTIAL TARGETS FOR THE TREATMENT OF TYPE II DIABETES

Guoqiang Jiang and Bei B. Zhang

Metabolic Disorders-Diabetes, Merck Research Laboratories, RY80N-C31, P.O Box 2000, Rahway, NJ 07065

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1. ABSTRACT

Type 2 diabetes is caused by a combination of impaired insulin secretion and, to a greater extent, resistance of target tissues to insulin action. Phosphoinositide 3-kinase (PI3K) plays a key role in insulin signaling and has been shown to be blunted in tissues of type 2 diabetes subjects. There is emerging biochemical and, particularly, genetic evidence suggesting that insulin resistance can potentially be treated via modulation of PI3K by targeting PI3K itself or its up and down-stream modulators. These potential targets include Src homology 2 domain containing inositol 5-phosphatase 2 (SHIP2), phosphatase and tensin homolog deleted on chromosome ten (PTEN), IκB kinase beta (IKK_β), PKC isoforms, and the PI3K p85 subunit. There is evidence suggesting that their inhibition affects PI3K activity and improves insulin sensitivity *in vivo*. In the current review, we will discuss the role of these molecules in insulin-mediated activation of PI3K, the rational for targeting these molecules for diabetes treatment, and some critical issues in terms of drug development.

2. INTRODUCTION

Diabetes mellitus is a disease defined by elevated levels of blood glucose, the homeostasis of which is maintained by the opposing regulation of insulin and glucagon. Lack of, or severe reduction in insulin secretion is responsible for insulin-dependent (type 1) diabetes mellitus. The defects in insulin secretion in type I diabetes are generally caused by autoimmune responses that lead to β-cell destruction. Non-insulin-dependent diabetes mellitus (NIDDM) or type 2 diabetes is generally caused by a combination of impaired insulin secretion and, to a greater extent, resistance of target tissues to insulin action. Major insulin responsive tissues include liver, muscle and adipose. Insulin resistance occurs when these tissues have attenuated response to circulating insulin. The detailed biochemical mechanisms for and the relative contribution of each of the insulin responsive tissues to the development of insulin resistance remain poorly understood. While certain single gene mutations (i.e., of insulin receptor) cause insulin resistance, they are rare. On the other hand, multifaceted genetic and environmental factors such as obesity associated with elevated circulating free fatty acid levels and hyperglycemia itself represent the more common risk factors that lead to or accelerate the development of insulin resistance and type 2 diabetes (1, 2).

To understand the mechanisms of insulin resistance and its treatment, one needs to have an understanding on the molecular mechanism of insulin signaling. As shown in Figure 1, it is generally thought that insulin binding leads to autophosphorylation and activation of the insulin receptor (IR), a transmembrane receptor protein tyrosine kinase. The phosphorylated-tyrosine residues in the activated IR protein provide docking sites for the binding of several down-stream signaling molecules such as the Shc, Grb2, and the insulin receptor substrate (IRS) proteins (IRS-1, 2, 3 and 4). The binding of these proteins leads to further activation of down-stream kinases

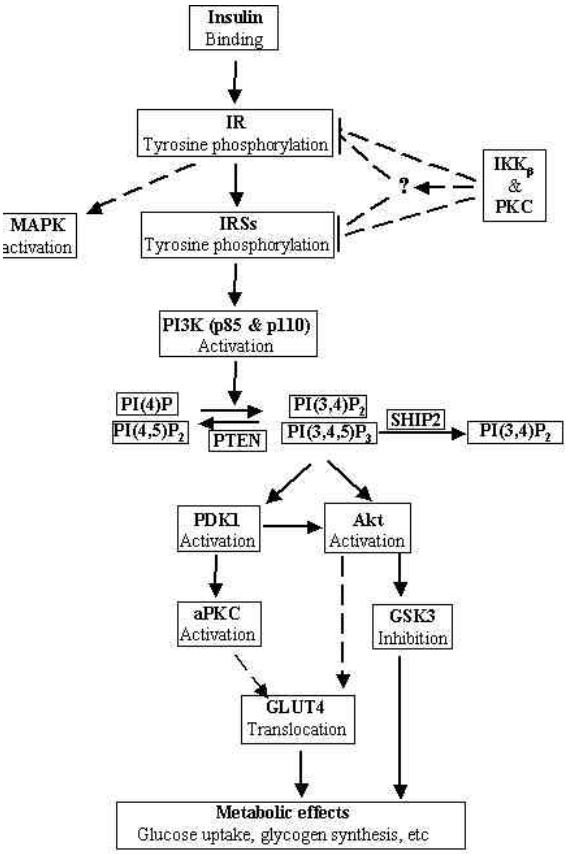

Figure 1. Elements of insulin signaling pathway and modulators. Shown is a rather simplistic view of the pathway depicting only the elements that are directly relevant to the discussions in the current review. IR: insulin receptor; MAPK: mitogen activated protein kinase; IRSs: insulin receptor substrate proteins including IRS1, 2, 3,and 4; IKK_β: IkB kinase β; PKC: protein kinase C; ?: unknown intermediate signaling molecule(s); PI3K: phosphoinositide 3-kinase; PI(4)P: phosphatidylinositide 4-phosphate; $PI(4,5)P_2$: phosphatidylinositide 4,5-biphosphate; $PI(3,4)P_2$: phosphatidylinositide 3,4-biphosphate; $PI(3,4,5)P_3$: phosphatidylinositide 3,4,5-triphosphate; SHIP2: Src homology 2 domain containing inositol 5-phosphatase 2; PTEN: phosphatase and tensin homolog deleted on chromosome ten; PDK1: PI3K-dependent serine/threonine kinase 1; aPKC: atypical PKC; GSK3: glycogen synthase kinase 3; GLUT4: glucose transporter 4. Arrow with solid line: direct interaction; Arrow with dotted line: interaction involving multiple intermediate steps or unclear mechanism(s).

including the mitogen-activated protein kinase (MAPK) and the phosphatidylinositol 3-kinase (PI3K). While the activation of MAPK is mostly involved in insulin-mediated mitogenic effects, the activation of PI3K is critical for insulin-mediated metabolic effects such as increased glucose uptake and glycogen synthesis (3-5).

The PI3K is a heterodimeric enzyme consisting of the p85 regulatory subunit as well as the p110 catalytic subunit. While there are three classes (type I, II and III) of PI3K based on different p110 isoforms and their substrate specificity, the current review will only focus on the class I (p110α, β, δ and γ), typified by the originally cloned p110α subunit. Activated PI3K specifically phosphorylates the D3 position of the inositol ring of the phosphatidylinositide (PI), phosphatidylinositide 4-phosphate (PI(4)P), and phosphatidylinositide 4,5-biphosphate ($PI(4,5)P_2$) to produce the PI(3)P, $PI(3,4)P_2$, and $PI(3,4,5)P_3$. $PI(3,4)P_2$ and $PI(3,4,5)P_3$ are evanescent phospholipids that are virtually absent in quiescent cells and rapidly up-regulated upon PI3K activation (6). Acting as second messengers, $PI(3,4)P_2$ and $PI(3,4,5)P_3$ recruit the phosphoinositide-dependent serine/threonine kinases (PDK1) and Akt from the cytoplasm to the plasma membrane. This occurs via binding to the "pleckstrin homology domain" (PH domain) of the kinases. Lipid binding and membrane translocation lead to conformational changes in Akt and to subsequent phosphorylation on Thr 308 and Ser 473 in the action loop by PDK1. Phosphorylation by PDK1 leads to full activation of Akt (4, 5, 7).

Activated Akt is known to phosphorylate and regulate the activity of many downstream proteins involved in multiple aspects of cellular physiology. Among others, Akt phosphorylates and regulates components of the glucose transporter 4 (GLUT4) complex, protein kinase C (PKC) isoforms, and glycagon synthase kinase 3 (GSK3), all of which are critical in insulin-mediated metabolic effects (3, 5, 7, 8). For instance, pharmacological inhibition of PI3K by wortmannin and LY294002 has been shown to block insulin-stimulated translocation of GLUT4 to cell surface and glucose uptake into cells (9-12). Overexpression of constitutively active forms of PI3K p110 catalytic subunit or Akt stimulates (8, 13, 14), whereas that of dominant-negative p85 regulatory subunit constructs blocks, insulin-mediated metabolic effects (15-20). While there has been some controversy regarding the role of Akt in insulin-mediated GLUT4 translocation (21), recent report that Akt2- but not Akt1-deficiency in mice is associated with insulin resistance and diabetes strongly supports the notion that Akt is important in insulin action (22, 23).

Given the central role of PI3K in insulin signaling, it is not surprising that insulin-stimulated PI3K and Akt activation has been found to be blunted in diabetic and insulin resistant states in both animals and man (24-33). Through direct or indirect fashions, the current therapeutics for type 2 diabetes potentiates insulin signaling to various degrees. By increasing circulating insulin concentrations, administration of exogenous insulin or promotion of the secretion of endogenous insulin (i.e., by sulphonylurea) lead to increased IR activation. Some other therapeutics (i.e., thiazolidinediones which are activators of the peroxisome-proliferator activated receptor gamma) increase insulin sensitivity *in vivo* (34-36). It has been shown that thiazolidinediones exert their insulin sensitizing effects at least partially by potentiating insulin-stimulated PI3K and Akt activation (32, 37-42).

There is emerging biochemical and, particularly, genetic evidence suggesting that insulin resistance can

potentially be treated via modulation of PI3K by targeting PI3K itself or its up and down-stream modulators. These potential targets include Src homology 2 domain containing inositol 5-phosphatase 2 (SHIP2), phosphatase and tensin homolog deleted on chromosome ten (PTEN), IκB kinase beta (IKK_β), PKC isoforms, and the PI3K p85 subunit. For each of these potential targets, there is evidence suggesting that their inhibition potentiates insulin signaling and/or improves insulin sensitivity probably by either increasing or, unexpectedly, decreasing the activity of the PI3K. The position of these signaling molecules in relation to the insulin signaling pathway is illustrated in Figure 1. In the current review, we will discuss the role of these molecules in insulin-mediated activation of PI3K, the rational for targeting these molecules for diabetes treatment, and some critical issues in terms of drug development.

While attempting to review the advances in the field, we are aware that it is only recently that these molecules have been linked to diabetes treatment and that the views on their potentials as therapeutics are likely to evolve and to be redefined fairly rapidly within the scientific community and pharmaceutical industries. This review will not cover the use of insulin receptor as a target for diabetes since it is a well validated target for existing therapeutics. However, it is worth pointing out that recent reports including ours indicate that, in addition to the use of natural or modified forms of insulin peptides, novel small molecule insulin mimics may represent potential new therapeutics (35). This review also will not cover GSK3 as a target for diabetes treatment since it is further downstream of the PI3K. Significant progress has been made recently on the signaling specificity of GSK3 in term of its role in cell growth vs metabolism. These findings suggest that one may be able to selectively modulate the metabolic but not the growth effects of GSK3 (43, 44).

3. SHIP2

SHIP2 is closely related to and, in fact, was cloned based on sequence homology to SHIP1 (45, 46). Structurally, both SHIP1 and SHIP2 proteins contain an SH2 domain in the N-terminus, an inositol-5-phosphatase domain in the center, a potential phosphotyrosine binding (PTB) domain binding site (NPAY) and putative SH3-domain binding moieties in the C-terminus. While both SHIP1 and SHIP2 specifically dephosphorylate the D5 position of the inositol ring, the suitable substrates must also have a phosphate in the D3 position. Though it remains controversial whether SHIP1 and SHIP2 dephosphorylate phosphatidylinositide 1,3,4,5-tetraphosphate $PI(1,3,4,5)P_4$, it is generally accepted that both dephosphorylate $PI(3,4,5)P_3$ (47, 48). While phosphorylation may affect the catalytic activity of SHIP1 and SHIP2, translocation via binding of the SH2 domain to phosphotyrosine in other signaling molecules is probably the key regulatory mechanism for these phosphatases (49, 50). SHIP1 has rather restricted expression pattern predominantly in the hemopoietic system. On the other hand, SHIP2 is ubiquitously expressed in multiple types of cells and tissues (45, 51, 52).

Given that SHIP2 dephosphorylates $PI(3,4,5)P_3$, one of the key products of PI3K, and that PI3K activation is critical in insulin signaling, it has been proposed that SHIP2 is a negative regulator of insulin signaling and that such negative regulation depends on its 5'-phosphatase activity. Several lines of evidence support such a proposal. Overexpression of SHIP2 protein decreases insulin-dependent $PI(3,4,5)P_3$ production as well as Akt and MAPK activation in CHO cells expressing a recombinant IR (53). Overexpression of SHIP2 protein also reduces $PI(3,4,5)P_3$ levels and Akt activity in EGF-stimulated COS-7 cells (49). It has also been shown that the overexpression of the wild type but not phosphatase-deficient SHIP2 protein inhibits insulin-stimulated Akt activation, GSK3 inactivation, and glycogen synthetase activation in L6 myotubes and in differentiated 3T3 L1 adipocytes, indicating that the inhibitory effects of SHIP2 on insulin signaling are lipid phosphatase activity-dependent. Finally, overexpression of SHIP2 protein does not affect insulin-stimulated IR tyrosine phosphorylation, IRS-1 tyrosine phosphorylation, and the association of IRS-1 with the p85 subunit of the PI3K, indicating that SHIP2 does not affect signaling components upstream of PI3K in the insulin signaling pathway (54, 55).

The potential of SHIP2 as a target for diabetes treatment was implicated by a recent study demonstrating that SHIP2-deficiency in mice increases insulin sensitivity *in vivo* (56). In this study, the SHIP2 gene was deleted by homologous recombination. It was shown that $SHIP2^{-/-}$ newborn mice have severe hypoglycemia and mortality. The mortality can be rescued by infusion of either glucose or an insulin neutralizing antibody. Tissues of the newborn knockout mice also have decreased expression of glyconeogenesic genes that could be normalized by the infusion of the insulin neutralizing antibody. Most interestingly, it was shown that, while $SHIP2^{+/-}$ mice do not have hypoglycemia and increased mortality, they have improved insulin sensitivity as indicated by improved glucose tolerance without concomitant increase in insulin levels as well as lower glucose levels in response to exogenous insulin. There is also an increased translocation of GLUT4 and increased glycogen synthesis in skeletal muscles. These results suggest that inhibitors of SHIP2 may represent a novel class of therapeutics for the treatment of type 2 diabetes by improving insulin sensitivity (57).

There are several important issues in targeting SHIP2 for diabetes treatment. $SHIP2^{-/-}$ mice have severe hypoglycemia and high mortality (56), suggesting that excessive inhibition of SHIP2 is undesirable. The question is how likely the severe phenotype of the $SHIP2^{-/-}$ mice is to occur in pharmacological inhibition. Since pharmacological inhibition is generally incomplete and occurs only in adult but not during embryonic development, there is a possibility that it may not lead to severe hypoglycemia and mortality as seen for the $SHIP2^{-/-}$ mice. The observation that $SHIP2^{+/-}$ mice have improved insulin sensitivity suggests that 50% or less inhibition of SHIP2 may be sufficient for improving insulin sensitivity

in vivo. At such levels, SHIP2 inhibition appears to be safe since $SHIP2^{+/-}$ mice are normal developmentally (56).

There is no increased tumorigenesis in SHIP2 deficient mice (56). This is somewhat surprising since deficiency of PTEN, the lipid phosphate that dephosphorylates the D3 position of the inositol ring of the $PI(3,4,5)P_3$, is strongly tumorigenic in multiple tissues and organs in both animal and human (58) (see more in next section for PTEN). There are potential explanations. First, PTEN dephosphorylates $PI(3,4,5)P_3$ and $PI(3,4)P_2$, both of which are produced by PI3K (59-61). On the other hand, SHIP2 only dephosphorylates $PI(3,4,5)P_3$ but not $PI(3,4)P_2$,. Since $PI(3,4)P_2$ has been shown to activate Akt as $PI(3,4,5)P_3$ does (62), the antagonizing effect of PTEN on PI3K is likely to be more complete than that of SHIP2. Therefore, PTEN deficiency is likely to activate Akt to much greater extents than SHIP2 deficiency does. Second, different from PTEN which is the only phosphatase known to dephosphorylate the D3-position of inositol ring, SHIP2 is a member of a family of enzymes that dephosphorylate the D5 position of inositol ring (63). Therefore, there may be compensation for SHIP2 but not for PTEN deficiency *in vivo*. The lack of tumorigenesis activity is crucial for diabetes therapeutics since such treatment is likely to be administered chronically.

It remains unknown how readily SHIP2 inhibitors can be obtained. So far, no potent and selective inhibitors for SHIP2 have been reported. Furthermore, given that the natural substrate $PI(3,4,5)P_3$ is highly charged, it is possible that competitive SHIP2 inhibitors, if found, will also be highly charged, resulting in limited ability to cross cell membrane and therefore reduced *in vivo* efficacy. It also remains to be seen whether one can obtain inhibitors selective for SHIP2 but not other 5-phosphatase of lipids particularly SHIP1. SHIP1 is expressed highly in hemopoietic cells and its deficiency has been shown to result in lymphocyte proliferation in mice (64). Given that SHIP1 and SHIP2 only share about 64% identity in the primary amino acid sequence in their catalytic domain (46), selectivity between SHIP2 and other 5-phosphatases may be obtainable. Given the uncertainty with small molecular inhibitors, it is possible however that alternative techniques such as antisense oligonucleotides to inhibit SHIP2 protein expression may be explored (65).

4. PTEN

PTEN was identified and cloned as a tumor suppresser gene found to be mutated in many animals and human cancers (58). The PTEN gene encodes a protein of 403 residues that shows homology to dual-specificity protein phosphatases. It contains the active site consensus motif HCXXGXXR(S/T) found in all protein tyrosine phosphatases. Although recombinant PTEN has been shown to dephosphorylate tyrosine-phosphorylated protein substrates, the physiological significance of such dephosphorylation remains controversial. On the other hand, there is clear evidence that PTEN dephosphorylates the D3 position in the inositol ring of the lipid second messenger $PI(3,4)P_2$ and $PI(3,4,5)P_3$ and that such dephosphorylation plays important roles in cell growth and survival (59-61). By dephosphorylating $PI(3,4)P_2$ and $PI(3,4,5)P_3$, PTEN antagonizes the action of PI3K, leading to reduced Akt activation (and other effects). PI3K and Akt activation play important roles in promoting cellular growth and survival. By inhibiting Akt activation, PTEN inhibits cell cycle progression and induces cellular apoptosis, at least partially explaining its role as a tumor suppresser (59-61, 66).

It was demonstrated that PTEN negatively regulates insulin signaling. In cultured cells, overexpression of PTEN protein has been found to inhibit insulin-induced $PI(3,4)P_2$ and $PI(3,4,5)P_3$ production (67-69), Akt activation (68, 69), GLUT4 translocation to the cell membrane (68 , 69, 70), and finally glucose uptake into cells (68). Additionally, microinjection of an anti-PTEN antibody increases basal and insulin-stimulated GLUT4 translocation (68). In contrast to the overexpression of the wild type PTEN, overexpression of catalytic inactive PTEN mutant does not negatively affect insulin signaling (69), indicating that lipid phosphatase activity is required for the action of PTEN on insulin signaling. Finally, it was reported that treatment with an antisense oligonucleotide which specifically inhibits the expression of PTEN (80% reduction in mRNA level in liver and adipose tissue) normalizes plasma glucose in the db/db mice *in vivo* (71). Taken together, these studies indicate that PTEN plays a negative role in insulin signaling and its inhibition improves insulin sensitivity. It therefore appears that PTEN may represent a novel mechanism for treating type 2 diabetes.

Successful targeting of PTEN for diabetes treatment should prove highly challenging on several fronts. As for SHIP2, no small molecule inhibitors have been found for PTEN in particular and for lipid phosphatases in general. Due to the charged nature of the natural substrates $PI(3,4)P_2$ and $PI(3,4,5)P_3$, competitive inhibitors for PTEN are likely to be highly charged, resulting in limited cell permeability and therefore *in vivo* efficacy. These problems, however, may be overcome via the use of antisense oligonucleotides as recently demonstrated (71). However, the biggest challenge lies in the fact that PTEN mutations are associated with many types of tumors in both animals and human (58). The observation that $PTEN^{+/-}$ mice are much more prone to tumorigenesis indicates that 50% inhibition of PTEN is sufficient to induce tumors (72-74). As discussed in the above section on SHIP2, the high tumorigenic activity for PTEN deficiency is at least partially due to the fact that PTEN dephosphorylates both $PI(3,4,5)P_3$ and $PI(3,4)P_2$ produced by PI3K and compensation for PTEN deficiency *in vivo* is unlikely to occur due to the lack of other related enzymes. Therefore, it remains to be seen that one can inhibit PTEN for improved insulin sensitivity without promoting tumorigenesis after long term treatment.

5. p85a

Three distinct genes encode the regulatory subunit of the PI3K: the $p85_\alpha$ gene, $p85_\beta$ gene and the $p55_\gamma$

genes. While the $p85_\alpha$ and the $p85_\beta$ proteins contain two SH2 domains and one SH3 domain, the $p55_\gamma$ protein does not contain the SH3 domain. The $p55_\gamma$ protein contains an unique 34 amino acid sequence at the N terminus (75-77). In addition to the $p85_\alpha$ protein, the $p85_\alpha$ gene also generates two splicing variants: the $p55_\alpha$ and the $p50_\alpha$ proteins both of which lack the SH3 domain and contain a unique 34 or 6 amino sequence at the N-terminus, respectively (78-80). All isoforms of the regulatory subunit have been found to associate with IRS proteins upon insulin stimulation (77, 80, 81). Given that, in insulin signaling, the association between p85 and the IRS protein precedes PI3K activation and that PI3K activation is essential for insulin-mediated metabolic effects, one predicts that p85 deficiency negatively affects insulin signaling and should lead to insulin resistance *in vivo*. Consistent with such a notion, overexpression of a domain negative form of the $p85_\alpha$ protein not only inhibits insulin-stimulated PI3K activation, but also Akt activation, glucose transport, glycogen synthase activation, and DNA synthesis in 3T3 L1 adipocytes (15).

Also consistent with the notion that p85 proteins are important in insulin action, several studies have demonstrated potential correlations between p85 gene polymorphism and insulin resistance. Nucleotide polymorphism (1020G-->A, changing a Met to Ile at codon 326) of p85 cDNA isolated from muscle has been found to be associated with significant reductions in whole-body glucose effectiveness, intravenous glucose disappearance constant, and insulin sensitivity index in Caucasians human subjects (82). The same polymorphism was also found to be prevalent in Japanese diabetic patient (83). In another study, a new variant (Arg409Gln) found in severe insulin resistant human subjects was shown to have slightly reduced ability to activate PI3K *in vitro* (84). Finally, variation in $p85_\alpha$ gene was also potentially linked to acute insulin response and type 2 diabetes in Pima women (85).

Somewhat unexpectedly, however, it is recently reported that deficiency of $p85_\alpha$ proteins may improve rather than decrease insulin sensitivity *in vivo* (86, 87). It was first reported that, in comparison with wild type mice, mice lacking only $p85_\alpha$ protein but still retaining $p55_\alpha$ and $p50_\alpha$ proteins ($p85_\alpha$-deficient mice) have normal steady-state plasma insulin but significantly lower steady-state plasma glucose levels. In the $p85_\alpha$-deficient mice, there is clearly an over-compensation by the $p50_\alpha$ alternative splicing isoform, resulting in actually higher levels of insulin-induced generation of $PI(3,4,5)P_3$ than in the wild type mice (86). Therefore, the results from the $p85_\alpha$-deficient mice are consistent with positive role of PI3K in insulin signaling. More recently, it is reported that mice deficient of all isoforms of $p85_\alpha$ ($p85_\alpha$, $p50_\alpha$, and $p55_\alpha$) ($p85_\alpha/p50_\alpha/p55_\alpha$-deficient mice) have much (~75%) reduced insulin-stimulated PI3K activity. Nevertheless, the $p85_\alpha/p50_\alpha/p55_\alpha$-deficient mice still appear to exhibit improved insulin sensitivity as indicated by reduced steady-state insulin levels, reduced steady-state glucose levels, and improved glucose tolerance (87).

The results from the $p85_\alpha/p50_\alpha/p55_\alpha$-deficient mice (87) suggest that down-regulation of PI3K by targeting $p85_\alpha$ protein improves insulin sensitivity *in vivo*, and may therefore represent another strategy for treating type 2 diabetes. There are several issues that one has to consider. The observation that $p85_\alpha/p50_\alpha/p55_\alpha$-deficient mice appear to have improved glucose metabolism (87) is consistent with the previous report that constitutive activation of PI3K results in insulin resistance in cultured cells *in vitro* (88, 89). The in vitro observations however is not consistent with the observation that $p85_\alpha$-deficient mice have increased PI3K activity and appear to have improved insulin sensitivity as well (86). Additionally, improved insulin sensitivity may not be the only explanation for the phenotype of the $p85_\alpha/p50_\alpha/p55_\alpha$-deficient mice as the authors indicated (87). Since these mice no only show hypoglycemia but also liver necrosis and perinatal death (87), they may be sick and therefore, by unidentified mechanism(s), exhibit abnormal glucose metabolism. The mechanism underlying the altered glucose metabolism in the $p85_\alpha/p50_\alpha/p55_\alpha$-deficient mice therefore remains unclear. Finally, since $p85_\alpha$, $p50_\alpha$, and $p55_\alpha$ proteins are not an enzyme, their inhibition will require approaches such as peptides to disrupt protein:protein interaction and antisense oligonucleotides to inhibit protein expression, both of which are theoretically possible but have not been extensively employed in the current therapeutics.

6. PKC ISOFORMS

Protein kinase C (PKC) is a family of structurally and functionally related proteins. To date, a total of 12 PKC isozymes (α, $\beta1$, βII, γ, δ, ε, η, θ, μ, ζ, and ι/λ) have been cloned and characterized. The PKC isoforms are subclassified into 3 major groups: the classical, novel and atypical PKCs. The classical PKC isoforms ($cPKC_\alpha$, $_{\beta1}$, $_{\beta II}$ and $_\gamma$) are activated by Ca^{2+}, phosphatidylserine, and diacylglycerols (DAG) (or phorbol ester). The novel PKCs ($nPKC_\delta$, $_\varepsilon$, $_\eta$, $_\theta$ and $_\mu$) are activated by phosphatidylserine and DAG but are independent of Ca^{2+}. The atypical PKCs ($nPKC_\zeta$ and $_{\iota/\lambda}$) are activated by phosphatidylserine but are independent of both Ca^{2+} and DAG. Activation of one or more PKC isoforms is involved in numerous cellular and physiological processes including insulin signaling. It has been shown that, upon insulin stimulation, DAG-insensitive $aPKC_\zeta$ and $aPKC_\lambda$ are activated in a PI3K-dependent fashion, and promote insulin-mediated translocation of GLUT4 to the plasma membrane. PI3K may activate aPKCs in both PDK1-dependent and independent fashion. Activated $aPKC_\zeta$ is associated with GLUT4 components and induces serine phosphorylation of the GLUT4-compartment-associated vesicle-associated membrane protein 2 (VAMP2) (90-98).

In contrast to the aPKCs, DAG sensitive cPKCs and nPKCs have been shown to inhibit insulin signaling by downregulating early signaling steps, leading to reduced PI3K activation. PKC phosphorylates and inhibits insulin receptor tyrosine kinase activity *in vitro* (99, 100). In various cell types, activation of several PKC isoforms (i.e., $PKC_{\beta I}$, $_{\beta II}$, $_\alpha$, $_\varepsilon$, and $_\theta$) by high glucose or phorbol ester is

associated with the inhibition of insulin receptor tyrosine kinase activity (101-104). The inhibitory effects of high glucose on insulin receptor tyrosine kinase activity can also be blocked by pretreatment with PKC inhibitors (101, 105, 106). The serine residues on insulin receptor important for the inhibitory phosphorylation of some PKC isoforms such as $PKC_{\beta 2}$ and $_{\theta}$ have been mapped (104, 107). Finally, PKC activation is also associated with reduced insulin receptor autophosphorylation *in vivo* in animals (108) and in human (109, 110).

In addition to phosphorylating insulin receptor, PKC isoforms have been shown to inhibit insulin signaling by promoting serine phosphorylation of IRS proteins such as IRS-1 (111-113). IRS protein serine phosphorylation has been shown to be associated with insulin resistance in multiple cell types (114-117). Upon serine phosphorylation, IRS-1 proteins have reduced ability to interact with insulin receptor, to be tyrosine phosphorylated by insulin receptor, and to bind PI3K (114, 118-121).

In animals and human, DAG is produced from hydrolysis of inositol phospholipids mediated by phospholipase C, hydrolysis of phosphatidylcholine mediated by phospholipase D, release of non-esterified fatty acids (NEFAs) from precursor lipids mediated by phospholipase A2, and *de novo* synthesis from phosphatidic acid (122). Type 2 diabetes patients are characterized by hyperglycemia and hyperlipidemia that have been shown to increase DAG levels and PKC activation. For instance, hyperglycemia induces DAG formation *in vivo* by promoting de novo synthesis and hydrolysis of phosphatidic acid (123, 124). *In vitro*, high glucose increases DAG level by also affecting the turnover of phosphatidylcholine (125, 126). Elevated levels of non-esterified fatty acids under hyperlipidemia also increase DAG. Corresponding to the elevated DAG level, DAG-responsive PKC isoforms have been found to be activated in the tissues of diabetic animals and human (123, 127-130).

The observation that DAG-responsive PKC isoforms are activated at elevated levels in diabetic subjects and that these PKC isoforms are capable of negatively regulating insulin signaling suggests that PKC inhibition may improve insulin sensitivity. However, several critical issues remain to be resolved. First of all, since many PKC isoforms have been found to be able to negatively regulate insulin signaling, the question is which isoform(s) of PKCs should be targeted. Several lines of evidence suggest that PKC_{θ} may represent a relatively promising target. Increased PKC_{θ} activation has been reported in the skeletal muscle of diabetic human and animals (110, 129, 131, 132). PKC_{θ} is activated during lipid infusion which resulted in insulin resistance in rats (133). PKC_{θ} is expressed predominantly (and almost exclusively) in skeletal muscle, hematopoietic tissues, testis and platelets. PKC_{θ} is the major isoenzymic form of PKC in skeletal muscle (134). The relatively restricted tissue distribution suggests that PKC_{θ} has relatively specialized physiological function and that its inhibition may also have relatively restricted physiological effects. Consistent with such a notion, $PKC_{\theta}^{-/-}$ mice are developmentally normal and fertile (135).

The PKC family is composed of 12 structurally related isozymes. Unlike some kinases (i.e., IKK_{β}) with restricted substrate specificity, PKC isoenzymes in general have broad substrate specificity. Therefore, any given isoform of PKC is likely to be involved in many aspects of cellular physiology, and simultaneous inhibition of multiple PKC isoforms is likely to produce undesirable side effects. Given that inhibitors for protein kinases in general have limited specificity (136), potent inhibitors highly selective for a specific PKC isoform may be difficult to obtain. Interestingly, however, it is reported that LY333531 is highly selective against $PKC_{\beta I}$ and $_{\beta II}$ and has been in clinical trails for retinopathy (137). Finally, while PKC activation has been associated with insulin resistance, there is a lack of strong and direct evidence that PKC inhibition improves insulin sensitivity *in vivo*. It is interesting to note that PKC_{β} knockout mice have enhanced overall glucose homeostasis *in vivo*, along with increased glucose transport in some tissues (138). It will be of great interest to test whether the deficiency of other PKCs (i.e., PKC_{θ}) improves insulin sensitivity *in vivo*.

7. IKK_b

IKK_{β}, a serine kinase, was purified and molecularly cloned based on its ability to phosphorylate IκB in response to cytokines including TNF_{α}. It is a component of a large IKK signalsome which also contains, among others, IKK_{α} and IKK_{γ}. Highly homologous to IKK_{β}, IKK_{α} is also a serine kinase and phosphorylates IkB. On the other hand, IKK_{γ} is a scaffold protein with no catalytic activity. It has been shown that IKK signalsome plays important roles in the signal transduction pathways leading to NFκB activation. In response to stimuli including cytokine, viral infection and stress, IKK_{α} and IKK_{β} become phosphorylated and activated. Activated IKK_{α} and IKK_{β} phosphorylate two serine residues in IκB. Serine phosphorylated IκB becomes ubiquinated and subsequently degraded via proteasome pathway. Prior to its degradation, IκB inhibits the activity of the nuclear transcription factor NFκB activity by directly binding to NFκB in the cytoplasm and preventing NFkB from translocating into the nucleus. After IκB degradation, NFκB translocates into the nuclears where it binds DNA and regulates the expression of many genes involved in immune and inflammatory responses. While both IKK_{α} and IKK_{β} phosphorylate and regulate the degradation of IκB at least in cells, gene knock-out experiments showed that IKK_{β} but not IKK_{α} deficient mice exhibit severe immuno depression. These studies suggest that IKK_{β} but not IKK_{α} is the key kinase in mediating IkB phosphorylation and NFκB activation *in vivo* (139, 140).

Salicylates including aspirin (acetylsalicylic acid) are anti-inflammatory drugs. It is generally thought that the anti-inflammatory effects of salicylates were mediated mainly by inhibition of cyclooxygenase-1 (COX1) and

cyclooxygenase-2 (COX2) (141). Given the important role of the IKK signalsome in immune and inflammatory response, it was also hypothesized that part of the anti-inflammatory effects of salicylates may be mediated via inhibition of IKK_α or IKK_β in addition to inhibition of COX1 or COX2. It was subsequently shown that, in *in vitro* phosphorylation experiments, salicylates inhibit the kinase activity of baculovirus-expressed recombinant IKK_β but not IKK_α in a dose-dependent fashion with an IC_{50} of 80 μM. The potency of aspirin in inhibiting IKK_β is inversely correlated with the concentration of ATP present, indicating a competitive mechanism (142). In cultured cells, salicylates also inhibit IkB phosphorylation (143), NFkB nuclear translocation and regulation of gene expression (144). In addition to salicylates, several other anti-inflammatory drugs have also been shown to inhibit IKK_β and/or IKK_α activity (145, 146).

It was reported that high does of salicylates including aspirin lower blood glucose concentrations. The earliest report on the anti-diabetic effects of aspirin was dated more than 100 years ago (see (147) for references). The observation that salicylates inhibits IKK_β and that salicylates may lower blood glucose led to the hypothesis that inhibition of IKK_β lowers blood glucose. This hypothesis was investigated in a series of experiments published recently (147, 148). These studies provide several interesting observations. First, in Zucker obese rats and ob/ob mice, long term (3-4 week) salicylate treatment improve glucose tolerance, reduce glucose levels in insulin tolerance test, and reduce triglyceride and free fatty acid levels (147). Second, short term (19 hr overnight) salicylate treatment also prevented lipid-induced insulin resistance (148). Third, $IKK_\beta^{+/-}$ mice have lower fasting glucose as well as lower fasting insulin on high fat diet, have improved glucose tolerance, and are resistant to lipid infusion induced insulin resistance. Finally, after being introduced into ob/ob mice, IKK_β deficiency but not COX1 or COX2 deficiency improves insulin sensitivity (147). Taken together, these results support the notion that IKK_β deficiency and its inhibition by aspirin improve insulin sensitivity *in vivo*.

As discussed previously, serine phosphorylation of both IR and IRS proteins has been associated with insulin resistance (99, 100, 114, 115, 149). Preliminary evidence suggests that IKK_β attenuates insulin signaling by facilitating serine phosphorylation of IRS proteins and probably insulin receptor as well, and that salicylates reverse these effects by inhibiting IKK_β (147, 148). This is likely due to an indirect effect, since IKK_β does not directly phosphorylate IRS-1 protein. It has been shown that PKC isoforms such as PKC_θ are upstream activators of IKK_β (150, 151). Since PKC isoforms have been shown to be activated *in vivo* in association with hyperlipidemia and/or hyperglycemia and elevated levels of DAG (123, 127-130), it is possible that, via PKCs, IKK_β may become activated in diabetic subjects.

The studies described above indicate that IKK_β represents a potential target for diabetes treatment. There are several favorable considerations for targeting IKK_β. First, results obtained with $IKK_\beta^{+/-}$ mice showing improved insulin sensitivity (147, 148) indicate that 50% inhibition of IKK_β may be sufficient for therapeutic effects. Inhibition at such levels appears to be safe since $IKK_\beta^{+/-}$ mice appear normal (152). Second, since $IKK_\beta^{+/-}$ mice have improved glucose tolerance without concomitant increase in insulin levels, the anti-diabetic effect of IKK_β inhibition is most likely mediated by insulin sensitization (147), representing an preferred mechanism for diabetes treatment. Third, so far, IKK_β appears to have unusually high substrate specificity. Except IκB, no other efficient substrates have been reported for IKK_β. It is therefore hopeful that specific IKK_β inhibition should produce relative restricted physiological effects. Fourth, IKK_β selective inhibitors have been reported. Compound SPC839 from Signal Pharmaceuticals has nM potency for IKK_β, but μM potency against IKK_α. More interestingly, the compound showed efficacy *in vivo* in models of chronic inflammation and in a model of septic shock and was found to regulate many immune genes in a DNA array study (153).

While IKK_β appears to be a potential target, there are also several important issues. First, there are reports suggesting that aspirin may exacerbate rather than improves insulin sensitivity (154). Second, the findings on the effects of aspirin were based on experiments in which the drug concentrations in the plasma reached several mM super-pharmacological levels (147, 148). As mentioned previously, aspirin inhibits the kinase activity of IKK_β but not IKK_α in *in vitro* phosphorylation experiments with an IC_{50} of ~80uM (142). This discrepancy suggests that the observed anti-diabetic effects of aspirin may be mediated via other alternative mechanism(s). Third, the detailed mechanism by which IKK_β inhibition improves insulin sensitivity remains unclear. Fourth, based on the observation that complete knock-out of IKK_β is embryonic lethal in mice due to hepatocyte apoptosis (152, 155), IKK_β inhibition may lead to liver damage. Additionally, since IKK_β is a critical component of the immune response, IKK_β inhibition may also lead to immunosuppression. Although $IKK_\beta^{+/-}$ mice appear normal developmentally (152), it remains to be seen whether these animals have comprised immunological responses, i.e., upon pathogen challenges. On the other hand, however, inflammation may be associated with diabetes (156-160). It is therefore possible that moderate immunosuppression, if achievable, may be beneficial for patients with diabetes.

8. CONCLUDING REMARKS

Given that type 2 diabetes is a fast growing health problem affecting increasing portion of world population and that its treatment have proven challenging particularly at the later stage of the disease, it is important to keep improving current therapies as well as developing new ones with novel mechanisms. In this review, we have discussed the components of the PI3K and its up- and down-stream modulators as potential targets for the treatment of type 2 diabetes based on both biochemical and genetic evidence. It is hopeful that, by targeting these

signaling molecules, we can treat type 2 diabetes by improving insulin sensitivity. There are however many challenges lying ahead. For instance, for lipid phosphatases such as SHIP2 and PTEN, no potent specific inhibitors have been reported. For IKK_{β} and PKC isoforms, the detailed mechanism(s) underlying their role in insulin action still remains largely unknown. For $p85_{\alpha}$, it remains to be seen whether its effects on insulin signaling are due to increased PI3K, decreased PI3K, or some other alternative mechanism. For all the putative drug targets discussed above, it remains to be seen that their pharmacological inhibition will improve insulin sensitivity but will not lead to unacceptable effects such as the potentiation of neoplastic transformation.

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Send correspondence to: Dr. Guoqiang Jiang, Metabolic Disorders-Diabetes, Merck Research Laboratories, RY80N-C31, P.O. Box 2000, Rahway, NJ 07065, Tel: 732-594-2176,Fax:732-594-3925,E-Mail: guoqiang_jiang@merck.com