Modulation of insulin signalling by insulin sensitizers

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Abstract
Insulin resistance is a hallmark of Type II diabetes. It is well documented that insulin sensitizers such as peroxisome-proliferator-activated receptor γ agonists and aspirin improve insulin action in vivo. The detailed mechanisms by which the insulin sensitizers promote insulin signalling, however, are not completely understood and remain somewhat controversial. In the present review, we summarize our studies attempting to explore the molecular mechanisms underlying the effects of insulin sensitizers in cells and in animal models of insulin resistance. In 3T3-L1 adipocytes and/or in HEK-293 cells stably expressing recombinant IRS1 protein (insulin receptor substrate protein 1), the peroxisome-proliferator-activated receptor γ agonist rosiglitazone and aspirin promote insulin signalling by decreasing inhibitory IRS1 serine phosphorylation. Increased IRS1 Ser-307 phosphorylation and concomitant decreased insulin signalling as measured by insulin-stimulated IRS1 tyrosine phosphorylation and Akt threonine phosphorylation were observed in adipose tissues of Zucker obese rats compared with lean control rats. Treatment with rosiglitazone for 24 and 48 h increased insulin signalling and decreased IRS1 Ser-307 phosphorylation concomitantly. Treatment of the Zucker obese rats with rosiglitazone for 24 h also reversed the high circulating levels of free fatty acids, which have been shown to correlate with increased IRS1 serine phosphorylation. Taken together, the results suggest that IRS1 inhibitory serine phosphorylation is a key component of insulin resistance and its reversal may be physiologically relevant to insulin sensitization in vivo.

Introduction
Insulin plays a central role in maintaining glucose homeostasis in both animals and humans. Upon the binding of insulin, insulin receptor undergoes autophosphorylation (exclusively tyrosine phosphorylation) and becomes activated. Through their Src homology 2 domain, IRS1–IRS4 (insulin receptor substrate proteins 1–4) bind to the phosphotyrosine residues in the insulin receptor, leading to phosphorylation of the IRS proteins on tyrosine residues. Tyrosine-phosphorylated IRS proteins provide further docking sites for downstream signalling molecules, including the p85 subunit of PI3K (phosphoinositide 3-kinase), resulting in the activation of a signalling cascade involving the activation of PI3K, activation of Akt, inactivation of glycogen synthase kinase, activation of glycogen synthase and translocation of GLUT4 (glucose transporter 4) from the cytoplasm to plasma membrane [1–3]. Although it has been long established that tyrosine phosphorylation of the insulin receptor and IRS proteins plays a positive and critical role in insulin signalling, more recent studies provide mounting evidence suggesting that serine phosphorylation of insulin receptor and IRS protein plays a critical but negative role in insulin signalling. In fact, serine phosphorylation of insulin receptor and IRS proteins represents one of the potential mechanisms responsible for the development of insulin resistance in vivo and its reversal could represent a potential target for the treatment of diabetes [4].

Serine phosphorylation inhibits insulin receptor autophosphorylation and activation [5–8]. On serine phosphorylation, IRS proteins have decreased ability to interact with insulin receptor, to be tyrosine-phosphorylated by insulin receptor kinase and to bind to PI3K [9–14]). Serine phosphorylation also promotes proteasome-mediated degradation of IRS proteins [14,15]. Multiple negative serine phosphorylation sites have been identified in both insulin receptor and IRS proteins. Increased serine phosphorylation of such sites has been found in cultured cells in response to a variety of treatments that impair insulin signalling [e.g. PMA and TNFα (tumour necrosis factor α)], in vivo in various tissues in response to certain acute treatments that induce insulin resistance (i.e. fatty acid infusion), and in vivo in various tissues under obese and diabetic states. Several kinases have been implicated in the negative serine phosphorylation of insulin receptor and IRS proteins. Among others, they include isoforms of protein kinase C (e.g. protein kinase Cθ), JNK (c-Jun N-terminal kinase) and IKKβ (IκB kinase β) [16–19].

In this paper, we summarize experimental results, mainly from our studies, suggesting that both PPARγ (peroxisome proliferators-activator receptor γ) agonists and aspirin...
potentiate insulin action by decreasing the serine phosphorylation of IRS proteins.

**PPARγ agonists decrease inhibitory serine phosphorylation of IRS-1 and insulin resistance**

PPARγ agonists such as TZDs (thiazolidinediones) are known to sensitize insulin action in *vitro* and *in vivo* and have been used for the treatment of insulin resistance in humans. While it is generally thought that activation of PPARγ is the predominant mechanism through which TZDs mediate their antidiabetic efficacy, the precise molecular events downstream of PPARγ activation that are critical for TZD-mediated insulin sensitization *in vivo* still remains somewhat unclear [20]. There is evidence suggesting that TZDs may exert their insulin-sensitizing effects, at least partially, by potentiating insulin signalling. For instance, it has been shown that insulin signalling is down-regulated in diabetic and insulin-resistant states in animals and humans and that such down-regulation can be reversed to various extents by treatment with TZDs [21–28].

We showed that the PPARγ agonist rosiglitazone (a TZD) potentiated insulin-stimulated Akt phosphorylation in HEK-293.IRS1 cells (HEK-293 cells expressing recombinant IRS1 protein) [29]. Similar results were observed on primary rat hepatocytes *in vitro* by both rosiglitazone and a non-TZD PPARγ agonist [30]. In Zucker obese rats, treatment for 24 h with rosiglitazone and a non-TZD increased insulin-stimulated tyrosine phosphorylation of insulin receptor and IRS1, and activates serine phosphorylation of Akt in both white adipose tissues and skeletal muscles. Treatment for 2–7 days, but not for 24 h, also increases insulin signalling in the liver [30]. The observation that two structurally distinct PPARγ agonists act in a similar manner to improve insulin signalling *in vitro* strongly supports the idea that the effects were indeed mediated by PPARγ. The observation that the potentiation of insulin action occurred in white adipose tissues and skeletal muscles after 24 h treatment but occurred in liver only after more than 48 h treatment suggests that the effects on the fat and muscle tissues may be primary.

Parallel to its ability to reverse PMA-induced insulin resistance in HEK-293.IRS1 cells, rosiglitazone was shown to decrease PMA-induced phosphorylation of IRS on Ser-307 and Ser-612 in the same cells. A similar observation was also made on differentiated 3T3-L1 adipocytes [29]. Furthermore, IRS1 Ser-307 phosphorylation was at a high level in the white adipose tissues of Zucker obese rats and such an increase was reversed by 24 h rosiglitazone treatment [29,30]. Taken together, these results suggest that IRS1 inhibitory serine phosphorylation is a key component of insulin resistance and its reversal contributes to the insulin-sensitizing effects by PPARγ agonists [29].

To understand how rosiglitazone treatment decreases IRS1 serine phosphorylation, we investigated the effect of rosiglitazone on several of the serine kinases that have been reported to phosphorylate IRS1. JNK phosphorylation (and hence activity) was found to be at a high level in the white adipose tissues of Zucker obese rats and such an increase was reversed after 48 h rosiglitazone treatment [29]. Rosiglitazone treatment has also been reported to decrease JNK1 phosphorylation in the heart of diabetic rats [31]. However, since the decrease of JNK hyper-phosphorylation in adipose tissues was not observed after 24 h rosiglitazone treatment when potentiation of insulin signalling was observed, it is unlikely that the insulin-sensitizing effect of rosiglitazone is primarily mediated by inhibition of JNK activity [29].

Similar to JNK, p38 MAPK (mitogen-activated protein kinase) was found to be hyper-phosphorylated (and hence activated) in the white adipose tissues of Zucker obese rats and such an increase was reversed by rosiglitazone treatment [29]. However, in contrast with the down-regulation of JNK after 48 h of rosiglitazone treatment, that of p38 occurred after 24 h of rosiglitazone treatment [29]. Therefore the concomitant down-regulation of the phosphorylation of IRS1 and p38 MAPK in the white adipose tissues of Zucker obese rats suggests a possibility that p38 has a role in the IRS1 serine phosphorylation and insulin sensitization by PPARγ agonists. In a different experiment, we also observed that p38 MAPK was hyper-phosphorylated in the liver of ZDF rats and such an increase was completely reversed by long-term treatment with another TZD PPARγ agonist, AD5075 (Figure 1A).

It has been reported that p38 promotes insulin-stimulated activation (not translocation) of GLUT4 and glucose uptake by muscle and fat cells [32]. In contrast with the down-regulation of p38 activating phosphorylation in white adipose tissues and liver as discussed above, up-regulation of p38 activating phosphorylation was observed in the skeletal muscles of Zucker obese rats after treatment with rosiglitazone for both 24 and 48 h (Figure 1B). It is therefore tempting to speculate that the up-regulation of p38 in the muscles may promote glucose uptake into muscles on treatment with rosiglitazone and other PPARγ agonists.

**Role of serine phosphorylation in insulin sensitization by salicylates**

Salicylates including aspirin (acetylsalicylic acid) have been shown to have antidiabetic effects in addition to being anti-inflammatory agents. Although such effects have been documented for more than a hundred years [33], more recent publications have provided evidence linking salicylates and insulin sensitivity. For instance, salicylates prevented lipid-induced insulin resistance in animals in short-term treatments [34]; salicylates improved insulin sensitivity and glucose homeostasis in long-term treatments in Zucker obese rats and *ob/ob* (obese–hyperglycaemic mice) mice [35] and Type II diabetic human subjects [36]. Furthermore, there is evidence suggesting that IKKβ, a serine/threonine kinase, promotes insulin resistance by serine phosphorylation of insulin receptor or IRS proteins and that salicylates improve insulin sensitivity by inhibiting IKKβ [33].

It has been shown that aspirin selectively inhibits IKKβ in a competitive manner *in vitro* [37]. IKKβ heterozygous mice have improved insulin sensitivity and are resistant to
lipid-induced insulin resistance [34,35]. While overexpression of active forms of IKKβ mutants led to insulin resistance, overexpression of a dominant inhibitory IKKβ mutant reversed insulin resistance in mice [38,39]. Finally, long-term treatment with parthenolide, another IKKβ inhibitor, was also found to improve insulin sensitivity in oβ/ob mice [40].

While the above studies are consistent with the proposal that IKKβ deficiency and its inhibition by aspirin improve insulin sensitivity in vivo, several questions remain unanswered. First, aspirin has been reported to exacerbate rather than improve insulin resistance in vivo [41]. Secondly, in the in vivo studies [34,35], the levels of salicylates in the blood reached several millimolar, much higher than the IC50 value for IKKβ inhibition determined in vitro. This raised the possibility that the antidiabetic effects of salicylates may not be entirely mediated by IKKβ. On another front, several recent studies suggest that IKKβ affects glucose metabolism by altering NF-κB (nuclear factor κB)-mediated transcriptional regulation of metabolically important genes such as GLUT4 instead of serine phosphorylation of insulin receptor or IRS proteins [42]. The detailed mechanism underlying aspirin-mediated metabolic effects is therefore not completely understood.

We showed that, in HEK-293.IRS1 cells, treatment with PMA and TNFα inhibited insulin-stimulated Akt phosphorylation while increasing IRS1 phosphorylation on Ser-307. Pretreatment with salicylic acids completely reverses the effect of both PMA and TNFα on the phosphorylation of both Akt and IRS1 [43]. A similar decrease in IRS1 serine phosphorylation of aspirin has been reported by others in vitro [44] and in vivo [35,45]. To understand how salicylic acid reverses PMA and TNFα-induced IRS1 serine phosphorylation in HEK-293.IRS1 cells, we also investigated the activation and potentially the function of several serine kinases in the cells.

In the HEK-293.IRS1 cells, IKKβ was activated by PMA but not by TNFα [43]. The lack of IKKβ activation by TNFα in the HEK-293.IRS1 cells is rather unexpected since IKKβ is normally activated by TNFα in various cells [46]. Nevertheless, the fact that IKKβ is not activated by TNFα excludes the possibility that the reversal of TNFα-induced insulin resistance and IRS1 serine phosphorylation by salicylic acid was mediated by IKKβ inhibition in this particular cell line. On the other hand, JNK was activated by both PMA and TNFα and such an activation was completely reversed by salicylic acid treatment. Furthermore, SP36600125, a JNK inhibitor, prevents PMA and TNFα-induced insulin resistance and IRS1 serine phosphorylation in the cells [43]. Accordingly, we proposed that aspirin acts to improve insulin sensitivity at least partially by inhibiting JNK activation and thus decreasing IRS1 serine phosphorylation [43]. Consistent with our proposal, aspirin was reported to reverse sepsis-induced insulin resistance, IRS1 serine phosphorylation and JNK activation in both muscle and white adipose tissues in septic rats in vivo [45]. Additionally, in a somewhat related fashion, aspirin was found to suppress the proliferation of metastatic B16 cells in a JNK-dependent manner [47].

There is ample evidence suggesting that JNK activation may lead to insulin resistance. JNK has been shown to associate with and to phosphorylate IRS proteins in vitro, resulting in decreased interaction between IRS proteins and the insulin receptor and, subsequently, insulin signalling [13,48]. JNK is activated under diabetic conditions which are associated with inhibition of insulin secretion [49]. JNK-deficient mice have decreased adiposity and improved insulin sensitivity [50]. Finally, a small molecule JNK inhibitor (CC105) was shown to improve glucose tolerance and increase insulin secretion in vivo in db/db mice. Consistent with the increased insulin secretion in vivo, islets isolated from oβ/ob mice treated with the inhibitor have much improved glucose-dependent insulin secretion ex vivo [19]. Taken together, JNK represents a potential target for the treatment of Type II diabetes [18,19,51].

**Concluding remarks**

Given that Type II diabetes is a fast growing health problem affecting an ever increasing portion of the world population and that its cure is still elusive, it is important that the
current therapies are improved upon and new treatments based on novel mechanisms are developed. Modulation of insulin signalling at various proximal and distal steps of the signal transduction pathway will hopefully yield new and improved insulin sensitizers that would become the mainstream for amelioration of insulin resistance and diabetes.

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