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REVIEW

RESISTIN ACTIVITY IN MICE AND HUMANS AFFECTING OBESITY, INSULIN RESISTANCE AND T2DM: BLOCKING RESISTIN ACTION BY RESISTIN ANTAGONIST

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Abstract

Resistin, that was originally discovered in 2001 is named for its capacity to resist insulin action shown mainly in mice. However, there is a controversial history regarding its role in the pathogenesis of insulin resistance and type 2 diabetes mellitus (T2DM) in humans as most of the research was based on association between resistin blood levels and T2DM or other pathologies and so far the pharmacological effect of recombinant resistin has not been tested in humans or even in primates. Resistin is a 12 kD cysteine-rich small protein acting as covalent dimer. Although human and mouse resistin genes and protein sequences share only approximately 60% homology, which is less than most hormones conserved across species, the genes are syntenic, with the mouse gene encoding resistin being located at a similar distance from the insulin receptor gene. Unlike mouse resistin which is mainly expressed in adipocytes, human resistin is synthesized predominantly in monocytes and macrophages, especially in the visceral adipose tissue. Resistin's effects are mediated via paracrine and endocrine mechanisms of action through a receptor on the surface of target cells that remains controversial. Others and our study has shown that resistin interacts with the Toll-like receptor 4 (TLR4), however other putative receptors such as an isoform of decorin, mouse receptor tyrosine kinase-like orphan receptor 1 (ROR1) and adenylyl cyclase-associated protein 1 (CAP1) have been also proposed. Downstream targets of resistin provide indirect evidence for its function in intracellular pathways, involving impairment of insulin signaling, response to inflammation and proliferation. Despite the lack of *in vivo* data concerning the resistin receptor and signaling in primates or humans, there is a need to explore the inflammatory, metabolic and oncogenic effects of resistin on human diseases. To address this challenge we have recently developed resistin mutant (C6A), acting as resistin antagonist which in several in vitro bioassays inhibited resistin action and in mice fed high fat diet (HFD) reduced weight, visceral fat, restored insulin sensitivity and attenuated HFD-induced neuroinflamation.

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Introduction

World Health Organization (WHO) has estimated that more than 347 million people worldwide are affected by diabetes and more specifically type 2 diabetes mellitus (T2DM). In addition WHO has also estimated that deaths due to diabetes will increase by two thirds between 2008 and 2030. Despite the enormous progress made to overcome this pathology, its progression worldwide is in permanent augmentation. Insulin resistance is one of the early features of T2DM, thus, the understanding of the mechanisms involved in the onset of insulin resistance is crucial for an early intervention circumventing then the establishment of T2DM. Insulin resistance is associated to several metabolic diseases and obesity (1). Obesity is now considered as a global epidemic affecting both adults and children, and is associated with significant morbidity and mortality rate and is caused by combination of genetic and environmental factors, including increased caloric intake, sedentary lifestyle and unhealthy eating habits. The prevalence of obesity among children, adolescents and adults has been dramatically increased during the last several decades. Worldwide, close to two billion adults are overweight and over 500 million are obese. According to several epidemiological studies, excessive body weight gain and obesity constitute a serious risk factor for insulin resistance and type 2 diabetes. It is also noteworthy that epidemiological studies reported that neurodegenerative diseases risk such as Alzheimer's disease (AD) is significantly increased 50%-100% in diabetic cases (2-4). More importantly, insulin resistance is now considered as an important etiological factor for AD because it is associated with reduced insulin-induced activation of insulin receptor and subsequent signaling pathways. Thus, the brain insulin resistance appears to be a common feature of AD constituting most likely an early event and marker of the disease. In addition, insulin resistance and T2DM are associated with inflammation and neuroinflammation that further worsen insulin responsiveness. Therefore, developing new therapeutic tools to fight insulin resistance is of high priority due to its dramatic consequences worldwide.

Resistin and insulin resistance

Insulin resistance is considered as a common hallmark of many metabolic disorders such as T2DM, obesity, dyslipidemias, inflammation, and cardiovascular diseases (1, 5-7). A tremendous number of publications have treated this issue in different tissues and cellular models. One of the puzzling issues concerns the potential link between obesity, inflammation and insulin resistance. Despite the very rich literature concerning insulin resistance, the molecular and cellular mechanisms linking obesity and inflammation to insulin resistance are not fully understood. More especially, how excessive body weight gain progressively promotes insulin resistance is a matter of controversy. Accumulating evidence however suggest that the alteration of whole body insulin responsiveness is initiated in the brain and more precisely in the hypothalamic nuclei, as hypothalamus is the main brain location controlling energy homeostasis by the integration of hormonal and metabolic signals responding to the energy body requirements. These signals mainly originate from adipose tissue (adipokines) and pancreatic β cells (insulin). Leptin, adiponectin and resistin are the major adipokines implicated in the regulatory loop involving adipose tissue and the hypothalamus to modulate energy homeostasis. These adipokines are also strong modulators of insulin responsiveness and inflammation at both central and peripheral levels. Among these adipokines, resistin is described as a potential factor in obesity-mediated insulin resistance, T2DM, inflammation (6, 7). We have recently reported that chronic ICV resistin infusion deeply impairs insulin responsiveness and signaling in the hypothalamus and peripheral tissues (adipose tissue, liver, muscle) in rats. Additionally, central resistin activates the serine kinases Jun NH(2)-terminal kinase (JNK) and p38 mitogen-activated protein kinase (p38 MAPK), enhances serine phosphorylation of insulin receptor substrate-1, and increases the expression of the pro-inflammatory cytokine interleukin-6 (IL-6) in the hypothalamus and key peripheral insulin-sensitive tissues (8). More importantly, we also reported the direct binding of resistin to Toll-like receptor 4 (TLR4) in the hypothalamus and showed that this binding activates proinflammatory pathways (8). Interestingly, resistin is able to induce the phosphorylation of Akt in neuronal cells *in vivo* (following ICV infusion) or in cell culture following short-term stimulation (10 to 20 minutes). In the same time, infusing resistin ICV during 14 days led to complete alteration of insulin-dependent Akt phosphorylation . This is explained by the deep alteration of insulin signaling as evidenced by the down-regulation of insulin receptor, the increase of serine phosphorylation of IRS1/IRS-2 and the induction of proinflammatory factors that are also involved in the onset of insulin resistance (Fig. 1). Furthermore, ICV infusion of resistin led to the down regulation of insulin receptor in liver, muscle and other tissues without significant changes in circulating resistin and this is explained by the vagal connexion linking the hypothalamus to the periphery. In term of signaling, Akt is down-stream of resistin/TLR4 signaling and also down-stream of insulin receptor/IRS signaling with probably different kinetics of action and also with different targets. Accordingly we hypothesized that resistin/TLR4 Akt pathway will target mainly gene expression whereas for insulin it will mainly target meta-

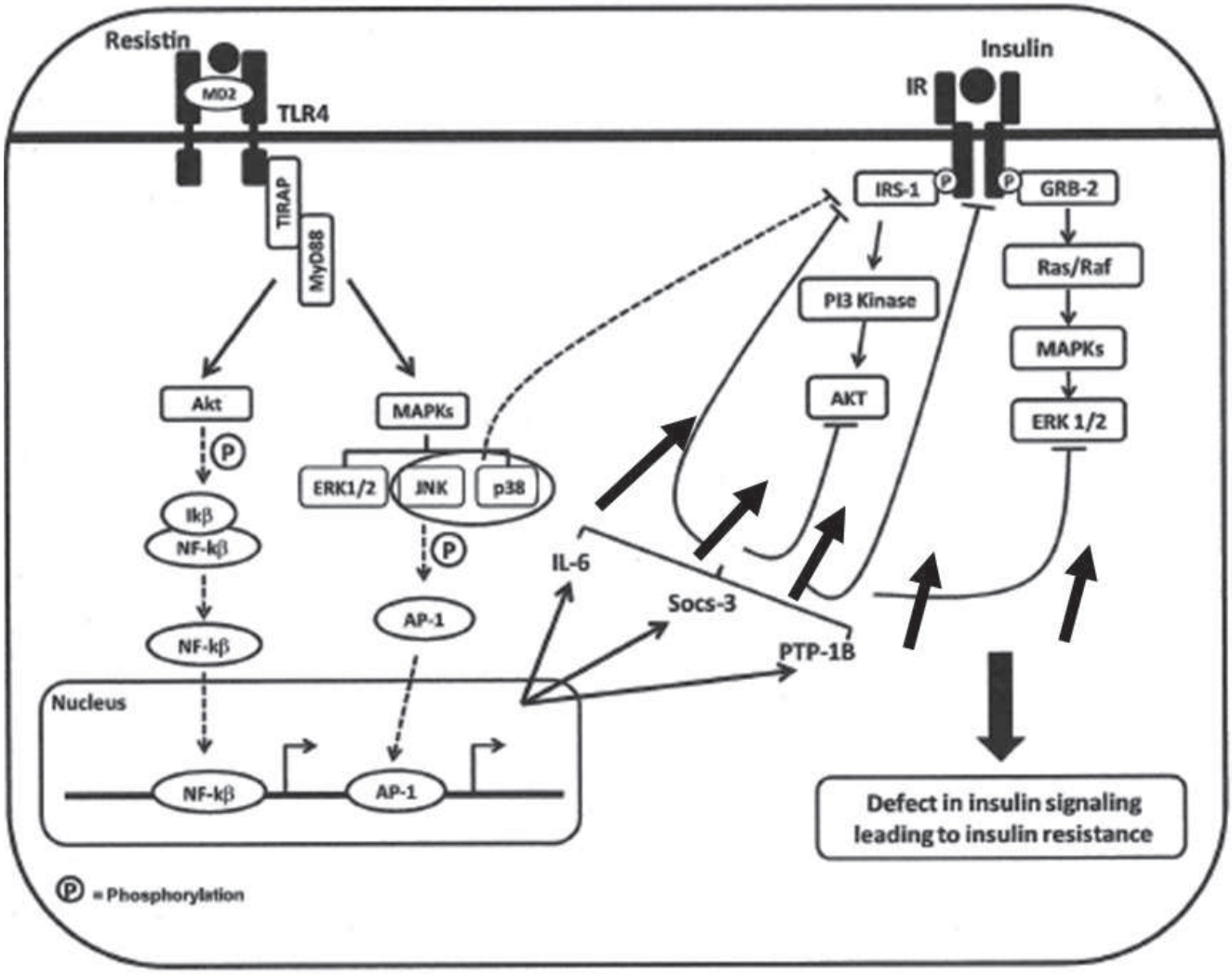

Figure 1. Schematic representation of resistin signaling that leads to inhibition of insulin signaling shown by 5 up-faced arrows: cytosolic activation of JNK and p38 kinases leading to serine phosphorylation and subsequent inactivation of IRS-1; nuclear activation of NF-kβ and AP-1 leading to enhance expression of IL-6, SOCS-3 and PTB-1B which in turn lead to attenuated activity of IRS-1, Akt, ERK 1/2 and dephosphorylation of insulin receptor followed by development of insulin resistance.

bolic actions. Consequently, we concluded that resistin/TLR4 signaling pathway seems to have a crucial role in the overall insulin-resistance and inflammation onset (8). Targeting this signaling pathway may constitute a significant breakthrough to overcome insulin resistance and inflammation, and related metabolic dysfunctions.

Comparative effects of resistin in mice and humans

Accumulating evidence indicates that changes in adipose-secreted factors in obesity, including release of inflammatory cytokines, dramatically affect insulin sensitivity (1, 5, 9-12). Among these adipokines, resistin is described as a potential factor in obesity-mediated insulin resistance and T2DM. Resistin is a cysteine-rich 12.5 kD polypeptide secreted by adipose tissue in rodents and by macrophages (mainly) in humans (10, 13), promoting inflammation and insulin resistance (14-17). Circulating resistin is increased in obese insulin-resistant rodents (12, 13) and humans (13) and fasting decreases resistin mRNA expression (12, 18). Peripheral administration or transgenic overexpression of resistin in rodents impairs insulin ac-

tion in insulin-sensitive tissues (19-21). Conversely, deletion of the resistin gene or infusing of resistin antibodies or antisense oligonucleotides restored insulin responsiveness (12, 22-24). More recently, it has been shown that resistin is expressed in the hypothalamus (25) and activates specific hypothalamic neurons (26). Central resistin also modulates glucose homeostasis, lipid metabolism, and food intake and impairs liver insulin sensitivity (27-30). Resistin also regulates the synthesis and secretion of key proinflammatory cytokines such as tumor necrosis factor-alpha, interleukin (IL)-6, and IL-12 in macrophages *via* a nuclear factor-kB–dependent pathway promoting insulin resistance (11, 12, 31, 32). However, whether the results obtained in mice are applicable to humans is still puzzling and controversial. Though some earlier publications questioned the link between resistin and insulin resistance in humans [see references 43-48 in (6)] our recent screening of the most recent 300 papers in PubMed under the term of resistin revealed 20 reports in which such an indirect link, evidenced by resistin levels in serum or tissues and various human pathologies such as adiposity, lung injury, metabolic syndrome, T2DM, gestational DM, diabetes-induced periodontitis, cardiovascular disease (CVD), non-alcoholic fatty liver disease (NFLD) and cancer was demonstrated (33-52). This issue was also widely addressed in the two most recent reviews. Abate *et al* (7) conclude that since inflammation plays a key role in the pathogenesis of T2DM (53) and resistin could be implicated in the development of T2DM, *via* induction of inflammation, adiposity or insulin resistance as shown in (54-56). In T2DM, macrophages, containing the highest levels of resistin gene expression (57, 58) that infiltrate the stroma of the adipose tissue and/or the vascular endothelium can locally produce and secrete resistin in addition to IL-1β, TNF-α, and IL-6, which may enhance the inflammatory load inducing insulin resistance and vascular dysfunction (58). Though macrophage infiltration into adipose tissue is a major feature of obesity, and macrophages are the main sources of resistin production, yet little is known about the differences in effects between serum and tissue resistin levels. Higher resistin levels have also been associated with T2DM complications, including renal dysfunction, atherosclerosis, gout, CVD, hypertension and peripheral neuropathy as shown in references 89-96 in Abate *et al* (7). Similar summary was reached by Park and Ahima in their recent review (6) who concluded: "Resistin was initially described as a link between obesity and insulin resistance in rodents. In rodents, resistin is almost exclusively expressed in white adipocytes, whereas human resistin is predominantly expressed in macrophages. The phenotypes of humanized resistin transgenic mice (59) suggest similar roles of murine and human resistin in insulin resistance. Studies in humanized resistin transgenic mice and epidemiological data also suggest that human resistin is a potential mediator between inflammation, and insulin resistance and atherosclerosis. Insights into the pathophysiological role of resistin will facilitate the development of novel diagnostic and treatment tools for diabetes, inflammation and CVDs".

Putative link between insulin resistance and resistin polymorphism in humans

Epidemiological studies conducted mainly in Japan linked resistin with insulin resistance and T2DM in humans. Polymorphism in the promoter of human resistin gene was positively associated with increased resistin levels in circulation (60). Particularly the -420 C>G (G/G) genotype at was associated with susceptibility to T2DM and also was correlated with monocyte resistin expression and with increased serum resistin levels (61, 62). Furthermore analysis of over 2000 Japanese subjects showed that plasma resistin was associated with SNP -420 and also correlated with insulin resistance (63). Similar results reported in a prospective study in China (64) and Egypt (65) but not in Korea (66) where the G/G (-420) genotype was associated with increased resistin levels but not with T2DM. In contrast, other studies were less conclusive: an Italian study showed that the presence of -420 C/G SNP is associated with increased obesity and metabolic syndrome but not with resistin levels (67), in Caucasian nondiabetic subjects in North America resistin levels associated with G/G (-420) genotype but not with insulin resistance (68), wheras in another study no such association was detected (69) . So far the origin of those discrepancies remains not explained and requires further investigations.

Interaction of resistin with TLR4 and other putative receptors

The understanding the biological function of human resistin has been so far hampered by lack of information about its corresponding receptor and signaling mechanisms. In four recent reports several proteins have been suggested as potential receptors for resistin: a TLR4 (70), an isoform of decorin (71), mouse receptor tyrosine kinase-like orphan receptor 1 (ROR1) (72) and most recently adenylyl cyclase-associated protein 1 (CAP1) (73). It was reported that the latter directly binds to human resistin and elicits inflammatory effects in cultured human monocytes and in white adipose tissue (WAT) in humanized resistin mice in vivo. In contrast both decorin and ROR1 are only putative receptors for murine resistin, and none of these have been shown to mediate the inflammatory effects of resistin in humans. We have demonstrated that at the neuronal level in vivo in rats that TLR4 interacts with recombinant human resistin produced in our lab and its TLR4-mediated signaling in the hypothalamus

lead to the activation of Jun NH2-terminal kinase (JNK) and p38 mitogen activated protein kinase (MAPK) signaling pathways by the recruitment of the adaptor proteins myeloid differentiating factor 88 (MyD88) and Toll/interleukin-1 receptor domain containing adaptor protein (TIRAP), promoting overall inflammation and enhancing insulin resistance (8). We have also shown that in SH-SY5Y human neuronal cells siRNA-mediated TLR4 knockdown impairs both resistin-induced inflammatory response and insulin resistance (8). Moreover, recent studies from other labs have provided evidence for the contribution of TLR4 in the pathogenesis of obesity and insulin resistance. Saturated fatty acids activate both hypothalamic and peripheral TLR4 signaling, leading to proinflammatory cytokine production and endoplasmic reticulum stress. Conversely, TLR4 loss-of-function prevents saturated fatty acid-induced inflammation and insulin resistance (74-77). Resistin and TLR4 have been linked to a proinflammatory process in a human epithelial cell line in which resistin competes with lipopolysaccharide (LPS) for binding to TLR4 (70). In SH-SH5Y human neuronal cells we demonstrated that adiponectin exerts an insulin sensitizing effect by improving insulin signaling through recovering of IR expression and marked reduction of JNK and IRS-1 phosphorylations. Overexposure of SH-SY5Y cells to resistin induced the downregulation of APPL-1 and adiponectin receptors leading to the impairment of adiponectin signaling and knock down of TLR4 completely abrogated resistin effects on adiponectin signaling. This effect was also verified *in vivo* where 14 days ICV chronic resistin infusion of Wistar rats led to significant down-regulation of hypothalamic APPLL-1 (78). In addition, in rheumatoid arthritis disease, resistin has been shown to use the IGF-1R signaling pathway. In conclusion, though TLR4 seems to be the major resistin receptor, a puzzling situation in which resistin could potentially interact with different receptors depending upon cellular or *in vivo* model is still to be considerated.

Development of resistin antagonist

As formation of covalent resistin dimer C6 bisulfide (S-S) bond is crucial for its activity (79, 80) we speculated that mutation of C6A may convert resistin to an inactive monomer, or even to resistin receptor antagonist. The DNA encoding the ORF mutated at C6A was subcloned into pMon vector, and expressed as insoluble protein in E. coli (MON 105 strain) upon induction with nalidixic acid. Subsequently, insoluble inclusion bodies were prepared, solubilized in Tris-buffered guanidine in presence of reducing agent and refolded by 1:25 slow dilution into oxidizing solution containing urea, arginine and low concentration of cysteine at 4^{o}C. Then the monomeric fraction was isolated to purity by preparative size-exclusion chromatography. Once it's *in vitro* efficacy in abolishing resistin-dependent Akt and ERK ½ phosphorylation in two types of cells was demonstrated it was termed resistin antagonist (RA) (81). Subsequently RA treatment of HFD-mice succeeded to reverse completely insulin resistance, to reduce the weight mainly due to reduction of visceral fat, to reduce expression of inflammatory cytokine IL6 and to attenuate HFD-induced neuro-inflammation and gliosis. In contrast, RA injections to lean mice fed chow diet did not affect the sensitivity to insulin, and even slightly (statistically borderline) increased their body weight (81) . In view of those and others' results using C6S mutant in lean mice (13) it seems the RA may act differently in lean and HFD mice. The reason for this is not entirely clear but may be related to the higher levels of resistin in the later.

Conclusions and future directions

As many of the basic questions related to resistin role in insulin intolerance and metabolic syndrome remain unknown, development of potent competitive RAs may provide a potent research tool for the mechanistic study of resistin in non-genetically manipulated animals. Furthermore, such antagonist may be therapeutically employed to counteract resistin's pathogenic effects, such as those documented in metabolic syndrome, T2DM and neuroinflammation. Therefore, our main research objective is to develop a super-active resistin antagonist and to demonstrate its activity in various *in vivo* experiments. For such antagonist to be effective, its affinity to the resistin receptor (TLR4) must be considerably higher than that of the native resistin, resulting in effective competitive inhibition of resistin action *in vivo*. To achieve this aim it is needed to employ a high throughput technique, based on repetitive selection of randomly mutated RA molecule presented on a yeast surface display library and selected by high-affinity and avidity binding using repetitive rounds of cell sorting as was demonstrated in our preparation of super-active leptin antagonists (82). Furthermore to test the *in vivo* effectiveness of the RA and it high-affinity, putatively superactive RA there is a need to prepare its mono-pegylated analogue which will extend its *in vivo* half-life in circulation. Such development of the recombinant high affinity RA may provide new and significant research and therapeutic advantages: (*i*) high throughput screening for structurally important binding motifs will extend our knowledge about resistin-TLR4 interactions, and (*ii*) the high affinity RA is expected to become an important new research tool in the field of metabolic syndrome biology, by allowing for the first time for potent and reversible inhibition of resistin. *In vitro* studies using the new molecules will enable the mechanistic study of downstream resistin effects on a variety of cells and tissues. *In vivo*, the superactive RA will enable the sys-

tematic study by blocking of the effects of endogenous resistin in non-genetically manipulated animals. The high affinity binding of the superactive RA will enable to achieve effective peripheral and central resistin inhibition in tolerable antagonist doses, using non-genetically modified animal models. Also, (*iii*) such new knowledge will enable future utilization of RA to modulate pathologic situations in which local or systemic resistin activity has been convincingly shown to play pathological role such as cancer and CVD. Though the later aspects of resistin function are not covered in the present review, blocking resistin action maybe highly beneficial in several types of malignancy as summarized in recent reviews (83-86) and in ameliorating elevated serum low density lipoprotein and thereby atherosclerotic CVD in obese humans (87-89).

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