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A novel IKKβ inhibitor stimulates adiponectin levels and ameliorates obesity-linked insulin resistance☆

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Abstract

Adiponectin is an anti-diabetic and anti-atherogenic hormone that is exclusively secreted from fat cells. Serum adiponectin levels are reduced in obese patients and obese model mice, despite increased adipose tissue mass. Elucidation of the mechanism(s) by which plasma adiponectin levels are decreased in obese and diabetic patients would provide insight into the cause of obesity-induced diabetes and the development of therapeutic advances. In the present study, the regulation of adiponectin secretion was investigated using 3T3-L1 adipocytes and a diabetic-/obese-mouse model. A novel insulin sensitizer, IκB kinase β (IKKβ) inhibitor, ameliorated insulin resistance and up-regulated plasma levels of adiponectin without producing a significant change in body weight in KKA^{y} mice that were fed a high-fat diet. The IKKβ inhibitor cancelled the TNFα-mediated down-regulation of adiponectin secretion and simultaneously up-regulated the phosphorylation of Akt in 3T3-L1 adipocytes. Using dominant-negative mutants of Akt or PKCλ (downstream effectors of phosphoinositide 3-kinase), insulin-stimulated Akt activity was found to be important in the regulation of adiponectin secretion by insulin in 3T3-L1 adipocytes. These observations suggest that "insulin-stimulated Akt activity in adipocytes" may play an important role in the regulation of adiponectin secretion.

Keywords: Adiponectin; Akt activity; Adipocytes; IKKβ inhibitor

Adiponectin [1–4] is an anti-diabetic and anti-atherogenic hormone. Recombinant adiponectin ameliorated insulin resistance in obese- and diabetic-KKA^{y} mice and diabetic-lipoatrophic mice both of which had reduced plasma adiponectin levels [5]. A single injection of recombinant adiponectin abolished hyperglycemia by suppressing glucose production in ob/ob, NOD (non-obese diabetic) or streptozotocin treated mice [6,7]. Transgenic overexpression of adiponectin also ameliorated insulin resistance in ob/ob mice [8]. Adiponectin-stimulated the activation of 5′-AMP-activated protein kinase (AMPK) in skeletal muscle and liver tissue [9]. Activated AMPK increased fatty-acid oxidation and glucose uptake in myocytes, reduced the expression

☆ *Abbreviations:* IKKβ, IκB kinase β; IRS, insulin receptor substrate; TNF, tumor necrosis factor; PI3-kinase, phosphoinositide 3-kinase; AMPK, 5′-AMP-activated protein kinase; PPAR, peroxisome proliferator-activated receptor; HF, high-fat; DEX, dexamethasone; IBMX, 3-isobutyl-1-methylxanthine.

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of molecules involved in gluconeogenesis in the liver, and reduced the glucose levels in vivo [9,10]. In addition, the disruption of the adiponectin gene is known to cause insulin resistance [11,12].

Adiponectin is specifically secreted by adipocytes [1,2]. Serum adiponectin levels, however, were reduced in obese patients [12] and obese mice models [4,5], despite an elevated mass of fat cells. Adiponectin levels were also reduced in diabetic patients and patients with coronary artery disease [12]. Elucidation of the mechanism(s) by which adiponectin secretion is reduced in obese and diabetic patients would provide insight into the cause of obesity-induced diabetes and the development of therapeutic advances.

Adiponectin secretion is increased by treatment with thiazolidinediones (TZDs), synthetic agonists for peroxisome proliferator-activated receptor γ (PPARγ) both in vivo and in vitro [5,13,14]. We previously reported, however, that heterozygous PPARγ deficiency resulted in a significant increase in adiponectin and a simultaneous amelioration of insulin resistance in mice [15]. These findings raised the possibility that the plasma adiponectin level may be more closely related to insulin sensitivity than PPARγ activity in vivo [15].

Recently IκB kinase β (IKKβ) [16], a serine/threonine kinase, has been shown to act as a key downstream mediator in obesity-linked insulin resistance by intracellular fatty acid metabolites. High doses of aspirin or salicylate, which inhibit IKKβ activity [17], have been shown to reverse hyperglycemia, hyperinsulinemia, and dyslipidemia in obese rodents by sensitizing the animals to insulin signaling [18,19]. Moreover, the heterozygous deletion of IKKβ protected against the development of insulin resistance during high-fat feeding [18] or lipid infusion [19]. Whether the amelioration of insulin resistance by the inhibition of IKKβ is accompanied by an increase in adiponectin levels, however, has not been reported. We used a novel IKKβ inhibitor [20] as an insulin-sensitizing agent to elucidate whether the plasma adiponectin level was related to insulin sensitivity.

In this report, the regulation of adiponectin secretion was investigated in diabetic-/obese-KKA^{y} mice and 3T3-L1 adipocytes treated with the novel IKKβ inhibitor. Our results suggest that insulin-stimulated Akt activity in fat cells is an important parameter in the regulation of adiponectin secretion in vivo.

Materials and methods

Chemicals. IMD-0354 [20], an IKKβ inhibitor, was synthesized at the Institute of Medicinal Molecular Design (Tokyo, Japan). Dexamethasone (DEX), 3-isobutyl-1-methylxanthine (IBMX), insulin, and TNFα were purchased from Sigma Chemical (St. Louis, Missouri, USA). All other materials were obtained from the sources given [21].

Animals. KKA^{y} mice were purchased from Nippon CREA (Shizuoka, Japan). Six-week-old mice were fed powdered chow according to previously described methods [15]. IMD-0354 was suspended in 0.5% sodium methylcellulose and administered intraperitoneally once a day. The animal care and handling procedures were approved by the Animal Care Committee of the University of Tokyo.

Determination of plasma glucose, insulin, leptin, and adiponectin levels. Blood samples were collected from the tail vein. The plasma glucose level was determined using the glucose-oxidase method and a commercial kit (Glucose-C-test; Wako Pure Chemical Industries, Osaka, Japan). The plasma insulin level was determined using an insulin ELISA kit (Wako Pure Chemical Industries, Osaka, Japan). The plasma leptin level was measured using a leptin ELISA kit (R&D, USA). The plasma adiponectin levels and adiponectin levels in media were determined using a previously described immunoblotting procedure [22]. In brief, 0.5 μl of serum or 10 μl of culture media was subjected to SDS–PAGE and transferred to nitrocellulose membranes. The membranes were immunoblotted with anti-adiponectin antisera and exposed to X-ray film (Fuji Film) using an ECL Western blotting detection reagent (Amersham Biosciences).

Cell culture, induction of adipocyte differentiation, and adenovirus infection. 3T3-L1 cells were cultured in DMEM with 10% FCS, and the adipogenic differentiation was induced according to previously described methods [21,23]. In brief, cells were cultured on 12-well plastic dishes and propagated to confluence. Two days later, the medium was replaced with a standard differentiation induction medium containing 0.5 mM IBMX, 0.25 μM DEX, 10 μg insulin/ml, and 10% FBS; two days later, the medium was changed to a maturation medium containing 5 μg of insulin/ml and 10% FBS; the medium was renewed every other day thereafter. Fully differentiated 3T3-L1 adipocytes were infected with adenovirus expressing lacZ, a dominant negative mutant of Akt, or a dominant negative mutant of PKCλ.

Results

IKKβ inhibitor, which ameliorates systemic insulin resistance and glucose intolerance, increased plasma adiponectin levels in KKA^{y} mice fed an HF diet

IMD-0354, which was discovered by the Institute of Medicinal Molecular Design, is a novel IKKβ inhibitor [20]. IMD-0354 inhibits the phosphorylation of IκB and the nuclear translocation of nuclear factor-κB (NF-κB) [20]. IMD-0354 significantly reduces the infarction area/area at risk ratio and preserves the fractional shortening ratio [20]. Recent studies have suggested that the inhibition of IKKβ ameliorates insulin sensitivity [18,19]. We administered IMD-0354 to KKA^{y} mice fed an HF diet to examine whether it would act like an insulin sensitizer. When insulin tolerance tests were performed at day 7, the plasma glucose levels decreased significantly in KKA^{y} mice treated with IMD-0354 and fed an HF diet in an IMD-0354 dose-dependent manner (Fig. 1B), but no significant changes in body weight (Fig. 1A, left panel) or white adipose tissue mass (Fig. 1A, right panel) were observed. Although the plasma glucose levels in KKA^{y} mice treated with IMD-0354 and fed an HF diet ad libitum did not change significantly (Fig. 1B, time = 0 min), the plasma insulin levels in the same mice decreased significantly in a dose-depen-

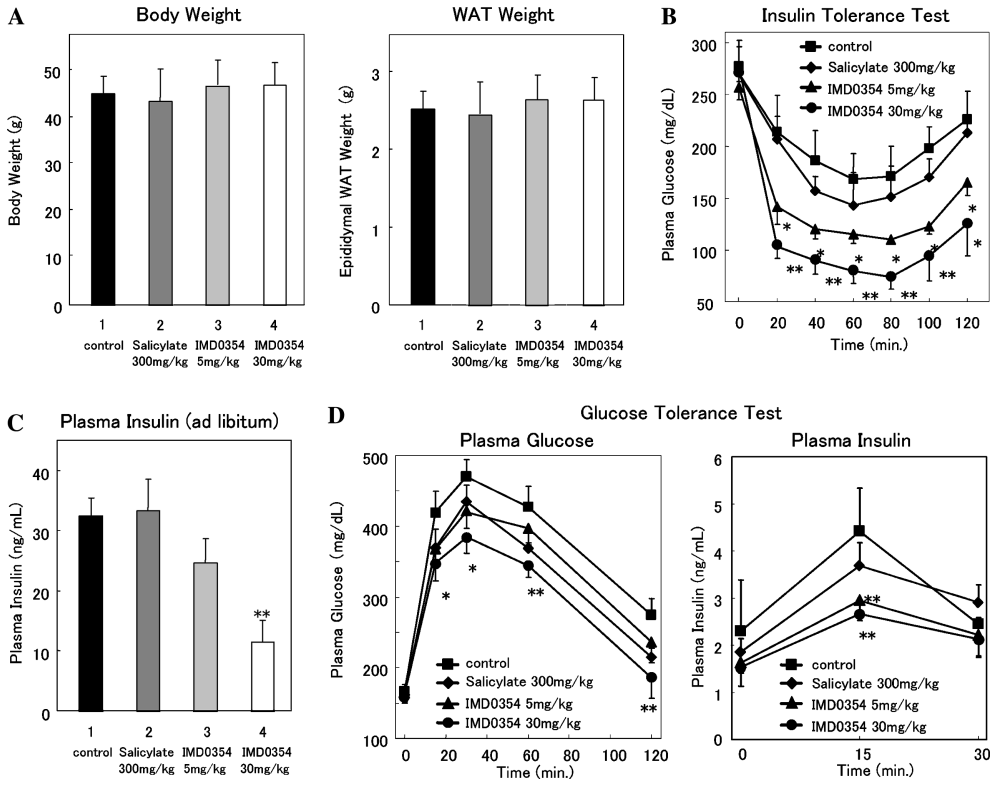

Fig. 1. An inhibitor of IKKβ (IMD-0354) ameliorates insulin resistance and glucose intolerance in KKA^y mice without producing changes in body weight or white adipose tissue weight. (A) Body weight and white adipose tissue (WAT) weight in KKA^y mice treated intraperitoneally with a vehicle, salicylate (300 mg/kg/day), IMD-0354 (5 mg/kg/day), or IMD-0354 (30 mg/kg/day) for 14 days. (B) Plasma glucose levels during insulin tolerance test (performed on day 7) in the same KKA^y mice shown in (A). Insulin (3 U/kg) was injected intraperitoneally. (C) Plasma insulin levels at 0 min (ad libitum) during the insulin tolerance test. (D) Plasma glucose and insulin levels during the oral glucose tolerance test (performed on day 10) in the same KKA^y mice shown in (A). Glucose (0.5 g/kg) was administered by oral gavage.

dent manner (Fig. 1C, lanes 3 and 4), suggesting that insulin sensitivity was increased in the KKA^y mice treated with IMD-0354. IMD-0354 was much more effective at ameliorating insulin sensitivity than salicylate (Figs. 1B and C, lane 2). When glucose tolerance tests were performed at day 10, the plasma glucose levels decreased significantly in KKA^y mice treated with IMD-0354 and fed an HF diet in a dose-dependent manner (Fig. 1D, left panel). Plasma insulin levels were also decreased in KKA^y mice treated with IMD-0354 (Fig. 1D, right panel). IMD-0354 was also much more effective at ameliorating glucose intolerance than salicylate (Fig. 1D). Moreover, the plasma adiponectin levels in KKA^y mice treated with IMD-0354 and fed an HF diet at day 7 increased in an IMD-0354 dose-dependent manner (Fig. 2A, lanes 3 and 4), while the plasma leptin levels were not altered (Fig. 2B).

These data suggested that (i) the IKKβ inhibitor ameliorated insulin resistance and glucose intolerance in a dose-dependent manner in KKA^y mice fed an HF diet more effectively and potently than salicylate, and (ii) either (a) the IKKβ inhibitor ameliorated insulin resistance and glucose intolerance by directly increasing the plasma adiponectin levels, or (b) the plasma adiponectin levels were indirectly increased because the IKKβ inhibitor improved systemic insulin sensitivity.

Insulin increased adiponectin secretion via the PI3-kinase-Akt pathway in vitro

We investigated the effect of IKKβ inhibitor on adiponectin secretion in cultured 3T3-L1 adipocytes to determine whether the increase in plasma adiponectin levels in KKA^y mice treated with an IKKβ inhibitor was a direct effect of the IKKβ inhibitor on adiponectin secretion and to identify possible effector(s) regulating adiponectin secretion. We also studied the effect of TNFα on adiponectin secretion because the expression of TNFα is known to be increased in adipose tissue in obese and diabetic patients [24] and is an important mediator of insulin resistance in subjects with obesity and diabetes through its ability to attenuate insulin receptor signaling via the inhibition of insulin receptor substrates (IRSs) [25,26].

In cell cultures of 3T3-L1 adipocytes, insulin increased the concentration of adiponectin in the media (Fig. 3A, lane 2) and TNFα decreased the insulin-induced elevation in the adiponectin concentration back

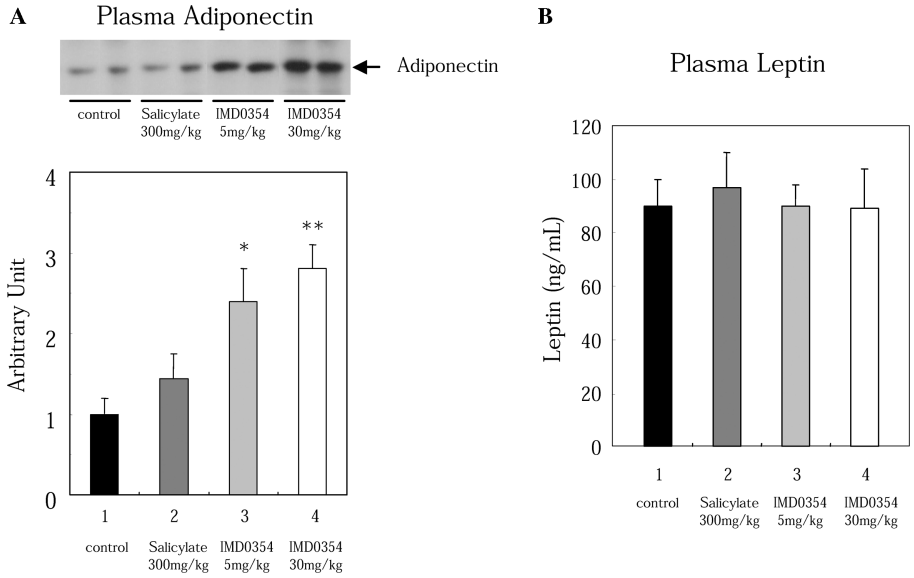

Fig. 2. An inhibitor of IKKβ increases plasma adiponectin levels in a dose-dependent manner in KKA^{y} mice fed a high-fat diet. (A) Plasma adiponectin and (B) plasma leptin levels in KKA^{y} mice treated intraperitoneally with the drugs shown in Fig. 1 at day 7. Each bar represents means ± SE. (n = 4–6). *p < 0.05; **p < 0.01.

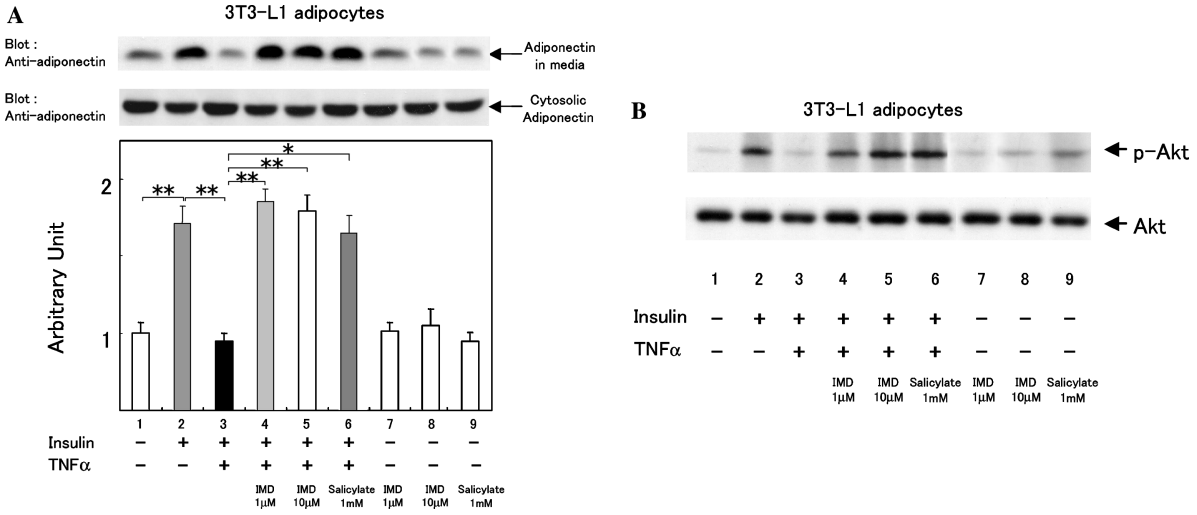

Fig. 3. Insulin increased adiponectin secretion via the PI3-kinase–Akt pathway in vitro. (A) Adiponectin levels in media (upper and lower panel) and cellular adiponectin levels (middle panel), and (B) the amount of phosphorylated Akt (upper panel) and the amount of Akt (lower panel) in 3T3-L1 adipocytes treated with insulin and/or TNFα and/or IMD-0354 or salicylate. 3T3-L1 adipocytes were serum-starved for 12 h and treated or not treated with drugs (salicylate or IMD-0354) and TNFα (6 nM) for 2 h. After a 12 h stimulation (A) or a 5 min stimulation (B) with 100 nM insulin, the media (A) and cells (A,B) were collected for immunoblotting. Each bar represents means ± SE. (n = 3). *p < 0.05; **p < 0.01.

to the basal level (Fig. 3A, lanes 1 and 3), in agreement with previous reports [3,13,27]. IMD-0354, an IKKβ inhibitor, ameliorated the TNFα-induced decrease in the adiponectin concentration in the media, when the TNFα and insulin were administered simultaneously (Fig. 3A, lanes 4 and 5). These changes were probably caused by increased adiponectin secretion because the cytosolic adiponectin concentration was nearly unchanged (Fig. 3A, middle panel). We also examined the phosphorylation of Akt, which is a downstream effector in insulin signal transduction; Akt phosphorylation is considered to be a marker of insulin signaling because previous studies have reported that TNFα causes insulin resistance via the down-regulation of Akt phosphorylation [28,29]. Insulin up-regulated Akt phosphorylation (Fig. 3B, lane 2), and TNFα down-regulated the insulin-induced up-regulation of Akt phosphorylation back to the basal level (Fig. 3B, lanes 1 and 3) in 3T3-L1 adipocytes. Furthermore, IMD-0354 restored the phosphorylation of Akt down-regulated by the TNFα

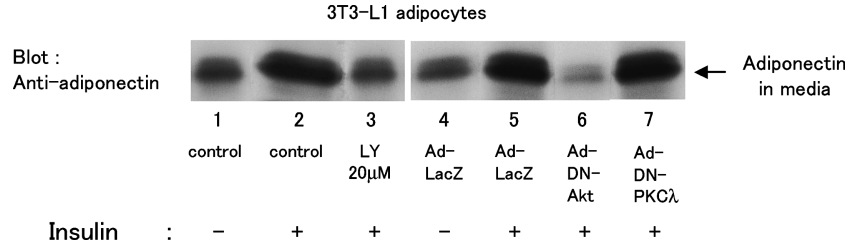

Fig. 4. PI3-kinase inhibitor and a dominant negative Akt mutant, but not a dominant negative PKCλ mutant, reduced the insulin-induced increase in adiponectin secretion in vitro. The amounts of adiponectin protein in the culture media were assessed by immunoblot analysis in 3T3-L1 adipocytes untreated (lane 1), treated with 100 nM insulin (lane 2), treated simultaneously with 100 nM insulin and 20 μM LY294002 (LY; an inhibitor of PI3-kinase) (lane 3), untreated after infection with an adenovirus expressing lacZ (Ad-lacZ) (lane 4), treated with 100 nM insulin after infection with Ad-lacZ (lane 5), or treated with 100 nM insulin after infection with an adenovirus expressing a dominant negative mutant of Akt (Ad-DN-Akt) (lane 6) or a dominant negative mutant of PKCλ (Ad-DN- PKCλ) (lane 7).

treatment, when TNFα and insulin were administered simultaneously (Fig. 3B, lanes 4 and 5). The administration of IMD-0354 alone had no effect on adiponectin secretion or Akt phosphorylation (Fig. 3A, lanes 7 and 8; B, lanes 7 and 8). These data suggested that the insulin-mediated increase in Akt phosphorylation and adiponectin secretion occurred in parallel.

PI3-kinase inhibitor and a dominant negative Akt mutant, but not a dominant negative PKCλ mutant, reduced the insulin-induced increase in adiponectin secretion in vitro

When LY294002, an inhibitor of PI3-kinase, was administered simultaneously with insulin, the adiponectin concentration of the media decreased (Fig. 4, lane 3), consistent with the findings of a previous report that insulin-induced PI3-kinase activation was involved in adiponectin secretion [27]. We next studied two major insulin-activated pathways (Akt and PKCλ), downstream of PI3-kinase to determine which pathway contributes to insulin-stimulated adiponectin secretion. The expression of a dominant negative Akt mutant by adenovirus infection also reduced the adiponectin concentration (Fig. 4, lane 6), while a dominant negative mutant of PKCλ had no effect (Fig. 4, lane 7). These findings suggest that insulin-stimulated Akt activity contributes to the positive regulation of adiponectin secretion by insulin.

Discussion

Reduction in plasma adiponectin levels is known to be an important cause of obesity-linked insulin resistance [11,12]. Although the agents that increase adiponectin levels are thought to be potential therapeutic insulin sensitizing drugs, no insulin sensitizers that increase the adiponectin levels have been reported except for TZDs.

Recently, two studies proposed that high-doses of aspirin or salicylate, which inhibit IKKβ activity [30], reversed hyperglycemia, hyperinsulinemia, and dyslipidemia in obese rodents by sensitizing the animals to insulin signaling [18,19]. However, the effect of the IKKβ inhibitor on adiponectin levels has not been reported. We examined the effect of an IKKβ inhibitor, IMD-0354 [20], on adiponectin secretion and obesity-linked insulin resistance, to verify whether insulin sensitivity regulates adiponectin secretion. The insulin-sensitizing effect of the novel IKKβ inhibitor, IMD-0354, was more effective and potent than that of salicylate in KKA^{y} mice fed an HF diet. These findings suggested that the IKKβ inhibitor increases adiponectin secretion either directly or indirectly via the amelioration of systemic insulin sensitivity. Consistent with the former possibility, IMD-0354 directly increased adiponectin secretion in 3T3-L1 adipocytes in an insulin-resistant state when TNFα with insulin was simultaneously administered. From in vitro data, the IKKβ inhibitor-induced increase in adiponectin secretion probably had a direct effect on adipocytes. Furthermore, these findings also suggested that IMD-0354 was a potential and a promising therapeutic agent for the treatment of high-fat diet-induced type 2 diabetes subjects with obesity.

Insulin has been shown to directly increase adiponectin secretion [3,27]. We verified the effect of insulin signaling on the increase in adiponectin secretion in 3T3-L1 adipocytes. IKKβ inhibitor ameliorated the reduction in the adiponectin concentration in the media of cell cultures where a state of insulin resistance had been induced by TNFα and simultaneously restored Akt phosphorylation in 3T3-L1 adipocytes. LY294002, a pharmacological inhibitor of PI3-kinase, decreased the concentration of adiponectin in media when administered simultaneously with insulin, consistent with the finding of a previous report [27]. We additionally studied the effect of Akt and PKCλ, two downstream effectors of PI3-kinase, using dominant-negative mutants of these molecules. Akt, but not PKCλ, played a causal role in the up-regulation of adiponectin secretion in insulin signaling, since a dominant negative mutant of Akt down-regulated the concentration of adiponectin while a dominant negative mutant of PKCλ did not.

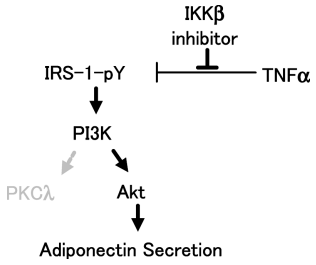

Fig. 5. Hypothetical mechanisms for the regulation of adiponectin secretion in vivo. Insulin-stimulated Akt activity in adipocytes is crucial to the regulation of adiponectin secretion (see Discussion).

The present investigations suggest that the IKKβ inhibitor suppresses the inhibitory effect of TNFα on IRSs and improves insulin signaling. Insulin signaling is transmitted from IRSs to PI3-kinase, an important mediator of glucose metabolisms. Furthermore, insulin signaling is transmitted to Akt, one of the major downstream pathways of PI3-kinase, and upregulates adiponectin secretion. In summary, insulin-stimulated Akt activity in adipocytes plays an important role in the regulation of adiponectin secretion (Fig. 5).

Acknowledgments

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