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Inhibition of the phosphatidylinositol 3′-kinase signaling pathway leads to decreased insulin-stimulated adiponectin secretion from 3T3-L1 adipocytes

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Abstract

Adiponectin is a protein secreted by adipocytes, which modulates insulin resistance and is thought to confer protection from atherosclerosis. Decreased circulating adiponectin is seen in states of insulin resistance, yet the cause of this decrease remains unclear. We investigated the role of insulin in adiponectin secretion and the effect of selective insulin resistance on insulin-stimulated adiponectin secretion by 3T3-L1 adipocytes. Inhibition of the phosphatidylinositol 3′-kinase (PI3K) insulin-signaling pathway was induced with wortmannin (WT) or with a kinase-inactive Akt adenoviral construct (Akt-KD), and inhibition of the mitogen-activated protein kinase pathway was induced with PD98059 or with a dominant-negative ras adenoviral construct (DNras). The PI3K pathway was activated with a constitutively active Akt adenoviral construct (Akt-myr). Adiponectin was measured by Western blot, and adiponectin messenger RNA (mRNA) levels were determined by real-time reverse transcription–polymerase chain reaction. Insulin treatment increased adiponectin secretion and decreased intracellular adiponectin. Treatment with 100 nmol/L insulin for 24 hours resulted in a 78% increase in secreted adiponectin ($P < .05$). Insulin had no effect on adiponectin mRNA. WT or Akt-KD, but not PD98059 or DNras, inhibited insulin-stimulated adiponectin secretion ($P < .05$). Activation of the PI3K pathway resulted in increased insulin-independent adiponectin secretion. Inhibition of the PI3K- or mitogen-activated protein kinase–dependent pathway decreased adiponectin mRNA by 50% ($P < .01$). We demonstrate a decrease in insulin-stimulated adiponectin secretion with selective inhibition of the PI3K pathway. These results suggest a mechanism for the observed decreased adiponectin levels associated with insulin resistance, when defects in the PI3K-dependent insulin-signaling pathway lead to decreased adiponectin production, inadequate adiponectin secretion, and therefore low circulating adiponectin levels.

1. Introduction

Adipose tissue, previously thought to be an inert storage depot for lipids, is now known to produce and secrete a variety of metabolically active proteins (“adipocytokines”) into the circulation [1-3]. One of these adipocytokines is adiponectin, a 30-kd protein exclusively produced and secreted by adipocytes [4-7]. Adiponectin has been found to be an important modulator of insulin sensitivity [8-10], and epidemiological studies suggest that high circulating levels of adiponectin may be protective against the development of coronary artery disease [11-14]. Low levels of adiponectin have been observed in both animals and humans with insulin resistance associated with obesity, diabetes, and lipody trophy [15-21] as well as in humans with coronary artery disease [11-14,22]. Although it appears that raising adiponectin levels in people with insulin resistance may be beneficial, a complete understanding of the mechanisms involved in adiponectin secretion and of the cause of low adiponectin levels in insulin-resistant states has yet to be achieved.

Insulin is a hormonal regulator of many secreted proteins, including adiponectin [23-25]. The mechanism of insulin action involves 2 main intracellular signaling pathways, which are activated when insulin binds to its cell-surface receptor [26,27]. One pathway, involving insulin receptor substrate proteins phosphatidylinositol 3′-kinase (PI3K) and Akt, is primarily responsible for the metabolic effects of insulin as well as for some anti-apoptotic and mitogenic effects [28-30]. In contrast, the pathway involving Shc, Ras,

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Raf, and mitogen-activated protein kinases (MAPKs) is responsible for numerous mitogenic effects of insulin and plays a small role in its metabolic action [31-33]. It is now recognized that in states of insulin resistance, insulin action is impaired along the PI3K intracellular signaling pathway [34,35], whereas signaling through the extracellular signal-regulated kinase MAPK pathway remains intact [35]. Therefore, in states of insulin resistance accompanied by compensatory hypersinsulinemia, molecular actions that occur through the PI3K pathway are inhibited, whereas those that occur through the intact MAPK pathway are either normal or augmented [30,36-38]. This selective inhibition of insulin signaling has been demonstrated in multiple different tissues including skeletal muscle [35], liver [39], vascular endothelium [36], and adipose tissue [40,41].

Although circulating levels of adiponectin [18,19,21, 42,43] as well as adiponectin messenger RNA (mRNA) [19] have been found to be diminished in the presence of insulin resistance, the regulation of adiponectin by insulin is not yet completely understood. Bogan and Lodish [25], as well as Motoshima et al [48], demonstrated an increase in adiponectin secretion in response to insulin stimulation. Published reports regarding the regulation of adiponectin expression by insulin are contradictory, with Fasshauer et al [23] showing a decrease in adiponectin mRNA and Halleux et al [57] an increase. Thus, in the present work, we examined both adiponectin production and secretion in the same cell system, as well as the effect of selective inhibition of the PI3K- and the MAPK-dependent insulin-signaling pathways on insulin-stimulated adiponectin synthesis and secretion. We hypothesized that insulin-stimulated adiponectin secretion and adiponectin expression would be inhibited by selective inhibition of the PI3K insulin-signaling pathway and that this signaling defect is responsible for decreased circulating adiponectin levels in states of insulin resistance.

2. Research design and methods

2.1. Cell culture

3T3-L1 pre-adipocytes (American Type Culture Collection, Rockville, MD) were cultured as per supplier instructions. Briefly, cells in equal amounts of cell suspension media were plated in 60-mm cell culture plates. Cells were grown to confluence in Dulbecco modified Eagle medium containing 1000 mg/L glucose, 10% fetal bovine serum, and antibiotics. Confluent pre-adipocytes were differentiated into adipocytes by growing in DMEM with 4500 mg/L glucose, 10% fetal bovine serum, antibiotics, 110 μg/mL isobutylmethylxanthene, 24.5 μmol/L dexamethasone, and 5 μg/mL insulin for 48 hours. The differentiation mix was replaced with adipocyte growth medium (AGM; DMEM with 4500 mg/L glucose, 10% fetal bovine serum, and antibiotics) plus 1 ng/mL insulin for 48 hours and then replaced every 2 days with AGM alone. Cells were allowed to fully differentiate in AGM for at least 96 hours before treatments. Cells were maintained in serum-free media for 24 hours immediately before treatments.

2.2. Pharmacological treatment

Fully differentiated serum-starved adipocytes were treated with pharmacological inhibitors of either the PI3K- or the MAPK-dependent pathway for 45 minutes before insulin treatment. The PI3K insulin-signaling pathway was inhibited with 100 nmol/L wortmannin (WT), and the MAPK-dependent insulin-signaling pathway was inhibited with 20 nmol/L PD98059. Adipocytes were then treated with 1, 10, or 100 nmol/L insulin for up to 36 hours. Platelet-derived growth factor (20 ng/mL) was used in the absence of insulin to test for specificity of the insulin effect.

2.3. Adenoviral infection

Recombinant adenovirus containing the complementary DNA encoding wild-type Akt (Akt-WT), Akt tagged with a myristoylation signal peptide sequence (constitutively active, Akt-myr), and kinase-inactive Akt (Akt-KD), kindly provided by Dr Kim Heidenreich, adenoviral construct encoding dominant-negative ras (DNras), kindly provided by Dr James DeGregori, and oncogenic H61-Ras construct, kindly provided by Dr Arthur Gutierrez-Hartmann, were used to selectively activate or inhibit the 2 main insulin-signaling pathways. Fully differentiated adipocytes were infected with each recombinant adenoviral construct at a multiplicity of infection of 50 to 100 for 16 hours. Transduced cells were incubated for 72 hours at 37°C, 5% CO_2, in DMEM high-glucose medium with 2% heat-inactivated serum, then in serum-free medium for 24 hours. The survival of 3T3-L1 adipocytes, as measured by total cellular protein, was unaffected by the different adenoviral constructs.

2.4. Western blotting

3T3-L1 adipocytes were lysed and sonicated. Twenty-five micrograms of each sample was dried using a Speedvac Concentrator (Savant, Holbrook, NY). Each sample was resuspended in 25 μL Laemmli (reducing buffer) and boiled for 10 minutes. Ten micrograms of total protein per lane was resolved by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride membranes. Membranes were probed with a mouse monoclonal adiponectin antibody (R&D Systems, Minneapolis, Minn), followed by a rabbit antimouse polyclonal antibody conjugated with horseradish peroxidase. Adiponectin was then detected by chemiluminescence (ECL Western Blotting Analysis System, Amersham Biosciences, Piscataway, NJ) and quantified by densitometry using a Bio-Rad Fluor-S MultiImager (Bio-Rad, Hercules, Calif).

2.5. Real-time reverse transcription–polymerase chain reaction

Intracellular adiponectin mRNA was measured by real-time reverse transcription–polymerase chain reaction (RT-PCR) amplification using the ABI 7700 Sequence

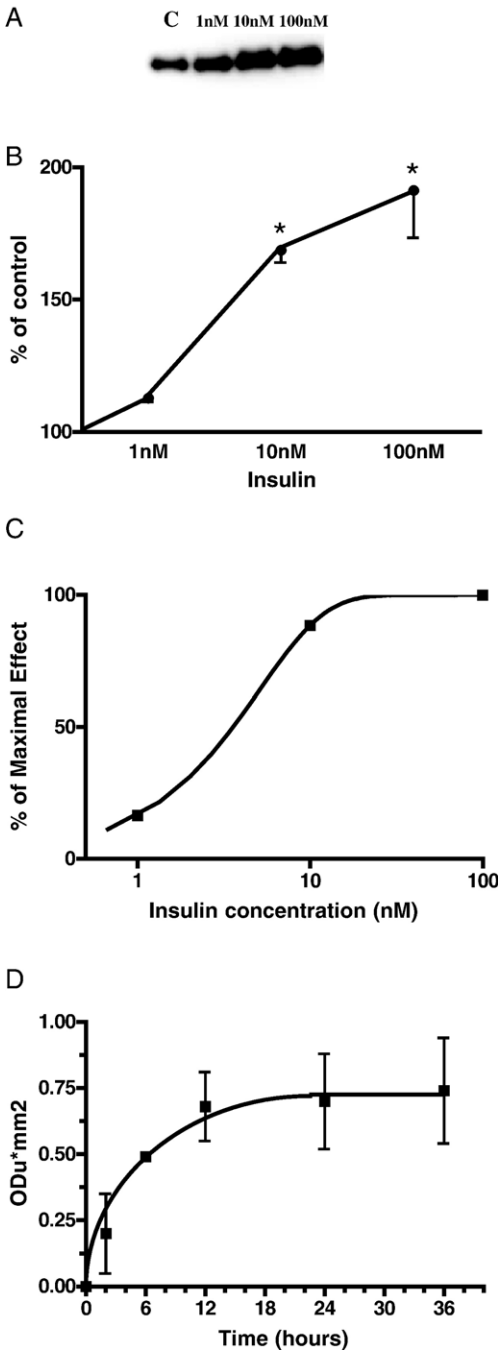

Fig. 1. Insulin stimulates secretion of adiponectin from 3T3-L1 adipocytes. Fully differentiated 3T3-L1 adipocytes were serum-deprived for 24 hours before treatment with various concentrations of insulin for 24 hours (A, B, and C) or with 10 nmol/L insulin for 2 to 36 hours (D). Protein secreted into culture media was extracted and separated by protein electrophoresis. Western blot membranes were probed with mouse adiponectin antibody and visualized using chemiluminescence as described in Research Design and Methods. Results are expressed as mean ± SEM of 5 to 8 experiments. Results plotted as percentage of the maximal insulin effect (C). Time course is plotted as densitometric units (ODu · mm^2) above untreated control for each period (D). *$P < .05$.

Detector (Applied Biosystems, Foster City, CA), with forward primer GGCCGTTCTCTTCACCTACG, reverse primer AGATGGAGGAGCACAGAGCC, and TaqMan probe TCAGGAAAAGAATGTGGACCAGGCCTC. Adiponectin levels were normalized to those of 18S.

2.6. Statistical analysis

Each experimental design was carried out 3 to 8 times, in duplicate. The data are presented as percentage of control and/or in densitometry units (optical density [ODu] · mm^2). The Student t test was used to determine the differences between the mean ± SEM of distinct treatment groups, with $P < .05$ considered statistically significant.

3. Results

3.1. Insulin stimulates secretion of adiponectin from 3T3-L1 adipocytes

We first examined the effects of insulin on adiponectin secretion into the culture media from 3T3-L1 adipocytes treated with increasing doses of insulin for 24 hours. Fully differentiated 3T3-L1 adipocytes were treated with 1, 10, and 100 nmol/L concentrations of insulin for 24 hours. Adiponectin secreted into the culture media was measured by Western blot as described in Research Design and Methods. Western blot analysis resulted in the visualization of one single band at 30 kd, corresponding to the expected weight

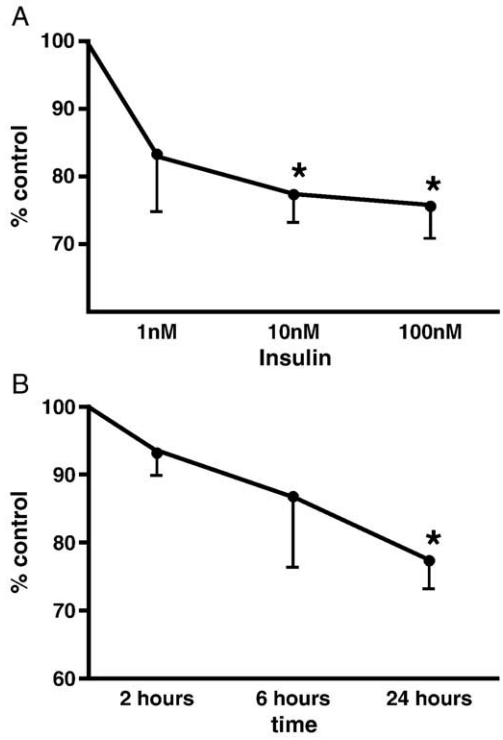

Fig. 2. Dose response and time course of insulin effect on intracellular adiponectin in 3T3-L1 adipocytes. Fully differentiated 3T3-L1 adipocytes were serum-deprived for 24 hours before treatment with various concentrations of insulin for 24 hours (A) or 10 nmol/L insulin for different periods (B). Intracellular adiponectin was determined by Western blot as described in Research Design and Methods and expressed as mean ± SEM of 3 to 5 experiments. *$P < .05$.

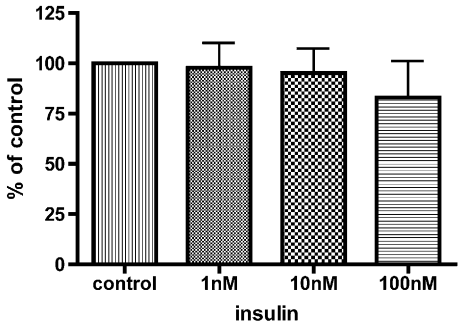

Fig. 3. Effect of insulin on adiponectin mRNA levels. Fully differentiated 3T3-L1 adipocytes were serum-starved for 24 hours and then treated with 1, 10, or 100 nmol/L insulin for 24 hours. Total RNA was isolated, and adiponectin mRNA was measured using the real-time RT-PCR technique. Results are expressed as mean ± SEM of 8 to 12 experiments.

of the adiponectin monomer. Insulin was found to increase adiponectin secretion in a dose-dependent manner, resulting in stimulation of 178% of control with 100 nmol/L insulin treatment ($P < .05$) (Fig. 1A and B). When the results are plotted as percentage of the maximal insulin effect (Fig. 1C), the half-maximal effect of insulin appears to be at a concentration of 4 nmol/L. A time-course study of adiponectin release demonstrated a leveling off of insulin-stimulated adiponectin release at around 12 to 18 hours, where insulin-stimulated release rate becomes equivalent to non–insulin-stimulated release rate (Fig. 1D).

3.2. Insulin decreases intracellular adiponectin in 3T3-L1 adipocytes

We then examined the effects of insulin on intracellular adiponectin in 3T3-L1 adipocytes treated with increasing doses of insulin for 24 hours. We expected the amount of intracellular adiponectin to decrease as insulin stimulated its release into the medium. Intracellular adiponectin was extracted and measured by Western blot as described in Research Design and Methods. Insulin treatment resulted in a significant and dose-dependent decrease in intracellular adiponectin ($P < .05$) (Fig. 2A). Insulin-stimulated decrease in intracellular adiponectin was also found to be time dependent, with continued decrease up to 24 hours ($P < .05$) (Fig. 2B).

3.3. Insulin has no effect on adiponectin mRNA levels

To determine whether the observed insulin-induced decrease in intracellular adiponectin is due entirely to insulin-stimulated secretion vs an inhibition of adiponectin production, we measured adiponectin mRNA by real-time RT-PCR. Insulin at doses of 1, 10, and 100 nmol/L had no effect on adiponectin mRNA levels (Fig. 3).

3.4. Insulin-stimulated adiponectin secretion is blocked by inhibition of the PI3K, but not by inhibition of the p44/42 MAPK insulin-signaling pathway

We then investigated the effect of selective insulin resistance on insulin-stimulated adiponectin secretion by using specific inhibitors of PI3K and the MAPK pathways. The PI3K insulin-signaling pathway was blocked using either the pharmacological agent WT or a dominant-negative Akt adenoviral construct (Akt-KD), and the MAPK insulin-signaling pathway was blocked using either the pharmacological agent PD98059 (PD) or a dominant-negative ras adenoviral construct (DNras). Pretreatment with WT but not PD was found to significantly inhibit insulin-stimulated adiponectin secretion ($P < .05$) (Fig. 4A and B). When comparing response to insulin in the setting of PI3K inhibition with WT vs response to insulin with no PI3K inhibition, insulin-stimulated adiponectin release decreased by about 25% with PI3K inhibition (1.1 ± 0.04 to 1.88 ± 0.12 ODu · mm^2 without WT vs 1.0 ± 0.11 to 1.63 ± 0.07 ODu · mm^2 with WT). In contrast, inhibition of the MAPK pathway using PD did not result in any significant change in insulin-stimulated adiponectin release.

Similar results were obtained with an adenovirus-mediated inhibition of these signaling pathways. Treatment with a kinase-inactive Akt construct decreased insulin-stimulated

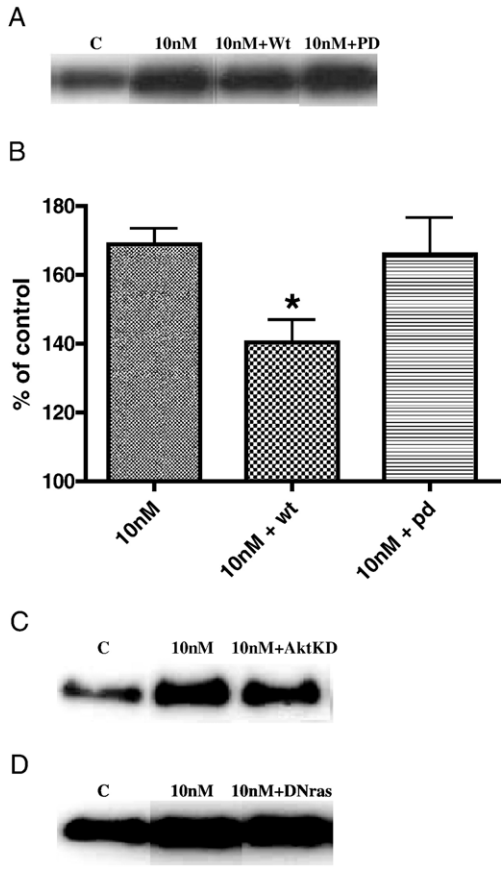

Fig. 4. Insulin-stimulated adiponectin secretion is inhibited by WT or Akt-KD, but not by PD98059 or DNras (A-D). Adipocytes were pretreated with WT or PD (A and B) for 45 minutes or infected with Akt-KD (C) or DNras (D) for 72 hours before treatment with 10 nmol/L insulin for 24 hours. Secreted adiponectin was measured by Western blot technique as described in Research Design and Methods. Results are expressed as mean ± SEM of 2 to 8 experiments. *$P < .05$.

adiponectin release (Fig 4C), whereas infection with a dominant-negative ras construct did not diminish the effect of insulin (Fig. 4D).

3.5. Adiponectin secretion is stimulated by adenovirus-mediated activation of the PI3K insulin-signaling pathway

Next, we examined adiponectin secretion in response to activation of the PI3K insulin-signaling pathway in the absence of insulin. 3T3-L1 adipocytes were infected with a recombinant adenoviral construct expressing constitutively active Akt. Adiponectin secretion into the medium was measured as described in Research Design and Methods. Adiponectin secretion into the media was increased by 36% to 40% with adenoviral activation of the PI3K pathway ($P < .05$) (Fig. 5). Platelet-derived growth factor in the absence of insulin had no effect on adiponectin secretion (data not shown), suggesting an insulin-specific effect. In addition, selective activation of the MAPK pathway using a constitutively active Ras construct (Ad-H61-Ras) in the absence of insulin did not affect adiponectin secretion (data not shown).

3.6. Basal adiponectin gene expression is suppressed by inhibition of either PI3K or p44/42 MAPK

In contrast to insulin, which did not influence adiponectin production, inhibition of either the PI3K- or the MAPK-dependent pathway in the absence of insulin significantly diminished basal production of adiponectin (Fig. 6). Both WT and PD98059 inhibited adiponectin mRNA levels by approximately 50%. Addition of insulin did not significantly change the observed decrease in adiponectin mRNA levels with WT or PD98059. Intracellular and extracellular adiponectin protein levels did not significantly differ from that of control after WT or PD treatment alone for 24 hours.

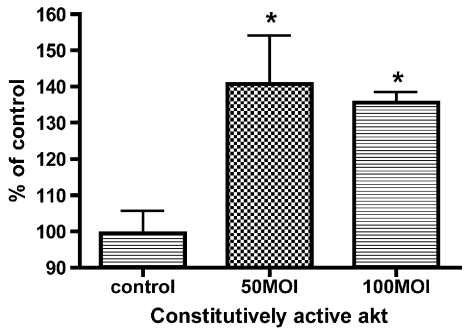

Fig. 5. Effect of activation of PI3K insulin-signaling pathway on adiponectin secretion. The PI3K insulin-signaling pathway was activated in the absence of insulin using a constitutively active Akt adenoviral construct (Akt-myr). Fully differentiated 3T3-L1 adipocytes were infected with Akt-myr adenovirus (50 or 100 multiplicity of infection) for 72 hours and then serum-starved for 24 hours. Adiponectin secreted into the medium for the next 24 hours was measured by Western blot technique as described in Research Design and Methods. Results are expressed as mean ± SEM of 2 experiments. *$P < .05$.

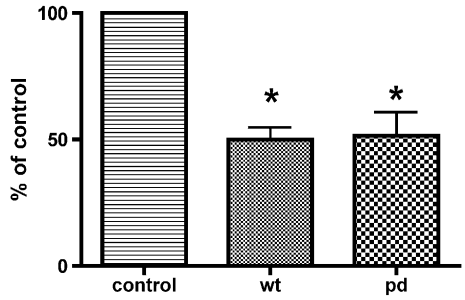

Fig. 6. Effect of WT and PD98059 on adiponectin mRNA levels. Fully differentiated 3T3-L1 adipocytes were serum-starved for 24 hours and then treated with 100 nmol/L WT or 20 nmol/L PD98059 for 24 hours. Total RNA was isolated, and adiponectin mRNA was measured using the real-time RT-PCR technique. Results are expressed as mean ± SEM of 7 experiments. *$P < .01$.

4. Discussion

Low circulating adiponectin levels associated with insulin resistance and hyperinsulinemia [18,21,44] may be responsible for an increased risk of atherosclerosis in patients with insulin resistance and diabetes [45-47]. However, the factors leading to low adiponectin levels in insulin-resistant states have not yet been determined. Because adiponectin production and secretion are performed exclusively by adipocytes, we studied the effects of insulin on these 2 processes in differentiated 3T3-L1 adipocytes. We investigated the effects of insulin on adiponectin production and release by treating 3T3-L1 mouse adipocytes in culture with increasing concentrations of insulin. We examined the signaling pathways involved in insulin regulation of adiponectin by pretreating cells with either WT, an inhibitor of the PI3K-dependent pathway, or PD98059, an inhibitor of the MAPK-dependent pathway. In addition, adenoviral constructs were used to selectively block (Akt-KD, DNras) or activate (Akt-myr, Ad-H61-ras) each insulin-signaling pathway in the absence of insulin.

We found that insulin stimulates adiponectin secretion in a dose-dependent manner and thereby reduces the levels of intracellular adiponectin. The half-maximal effect of insulin is seen at 4 nmol/L, a high physiological concentration of this hormone. Several other investigators have also described the stimulatory effects of insulin on adiponectin release in vitro. Bogan and Lodish [25] conducted pulse-chase experiments using 3T3-L1 adipocytes and demonstrated that adiponectin is sorted into insulin-regulated secretory vesicles as well as non–insulin-stimulated constitutively active secretory vesicles. Motoshima et al [48] have reported insulin-stimulated adiponectin release from cultured human omental adipocytes. Our data support these previous studies and provide further evidence for insulin's stimulatory effects on adiponectin secretion via the PI3K-dependent signaling pathway.

The mechanism of insulin action involves an activation of the PI3K and the MAPK-dependent pathways [34,49]. The former intracellular signaling pathway appears to be responsible for the metabolic effects of insulin in various tissues [28,50], stimulation of nitric oxide production by endothelial cells [29], and maintenance of the differentiated state of vascular smooth muscle cells [30]. In contrast, activation of the MAPK-dependent pathway has little effect on the metabolic insulin action, but is responsible for numerous mitogenic and nuclear effects of insulin [30-33,35,36,38]. It has been shown that in the presence of metabolic insulin resistance, the PI3K-dependent pathway is inhibited, whereas transmission of the insulin-induced signal along the MAPK-dependent pathway remains intact [29,30,35-38,51,52]. Furthermore, compensatory hyperinsulinemia, which arises in response to metabolic insulin resistance, can provide excessive stimulation of the MAPK-dependent pathway [29,30,38].

In the case of insulin's effect on adiponectin release, the signal generated by insulin appears to proceed via the PI3K-dependent pathway. We show that inhibition of the PI3K insulin-signaling pathway blocks insulin-stimulated adiponectin secretion, whereas selective activation of the PI3K pathway in the absence of insulin increases adiponectin secretion. Thus, in the insulin-resistant state, when the strength of signaling via this pathway is diminished, insulin-stimulated adiponectin release would be expected to decrease. Although the role of adiponectin secretion rate in maintaining adiponectin plasma levels in vivo has been questioned [55], our experiments suggest that long-term inhibition of adiponectin release in the presence of diminished insulin signaling may be a factor in the decreased adiponectin levels that accompany insulin resistance.

Decreased adiponectin levels have been observed in animal models of insulin resistance [6,53,54] as well as in humans with insulin resistance associated with obesity, type 2 diabetes, or lipodystrophy [15,18,19,42,55]. Furthermore, an improvement in insulin sensitivity in patients treated with insulin-sensitizing thiazolidinediones, or caused by weight loss, has been found to result in increased adiponectin levels, suggesting that insulin is a regulator of serum adiponectin levels [56]. Our finding that insulin stimulates adiponectin secretion by acting through the PI3K-dependent pathway provides a reasonable explanation for the low serum adiponectin levels observed in states of insulin resistance, where the PI3K-dependent pathway is blocked.

Because the decrease in intracellular adiponectin we observed with insulin treatment could also have been due to an inhibitory effect of insulin of adiponectin production, we measured adiponectin mRNA levels by real-time RT-PCR in cells incubated with increasing concentrations of insulin for 24 hours. In contrast to observations reported by Fasshauer et al [23] and Halleux et al [57], we did not find any effect of insulin on adiponectin production. An inhibitory trend observed at 100 nmol/L insulin remained nonsignificant. However, we did find that inhibition of either the PI3K- or the MAPK-dependent pathway (in the absence of insulin) significantly reduces production of adiponectin. These data indicate that both pathways must be intact for a normal production of adiponectin. Although we did not find that inhibition of either the PI3K- or MAPK-dependent pathway for 24 hours resulted in a significant change in intracellular or extracellular adiponectin levels in the absence of insulin, these pathways likely play an important role in the regulation of adiponectin levels in vivo.

We have shown that adiponectin secretion is enhanced by insulin action although adiponectin mRNA does not change. We have also shown that intact functioning of the insulin-signaling cascade is necessary for the maintenance of basal adiponectin mRNA levels. In vivo, in states of normal insulin sensitivity, steady-state adiponectin levels are likely maintained through a complex regulation of mRNA production and degradation, transcription of the gene, intracellular compartment trafficking, and secretion of adiponectin into the circulation. In the presence of insulin resistance, when the PI3K signaling pathway is inhibited, there would be a reduction in basal adiponectin production, contributing to lower levels of intracellular adiponectin. Combined with a reduced ability of insulin to stimulate adiponectin release, this would further contribute to low circulating levels of adiponectin in insulin-resistant states.

In summary, we find that insulin stimulates adiponectin secretion via the PI3K signaling pathway, and that both the PI3K- and the MAPK-dependent insulin-signaling pathways are necessary for basal adiponectin production. In insulin-resistant states, where there is a defect in PI3K-dependent signaling in the adipocytes, decreased adiponectin production in concert with inadequate secretion would be expected to result in low levels of circulating adiponectin. This finding suggests that medical therapies that improve insulin action at the adipose tissue level may be able to increase circulating adiponectin and may provide a potential strategy for decreasing atherosclerosis risk in patients with insulin resistance.

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