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Globular adiponectin activates Akt in cultured myocytes

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ABSTRACT

The serine/threonine kinase Akt plays an important role in insulin-mediated glucose uptake. Adiponectin (Adp) is known to sensitize this process. The purpose of the current study is to investigate if Adp activates Akt independently from insulin; and if Adp synergizes with insulin on Akt phosphorylation in the rat skeletal muscle L6 cells. Differentiated L6 cells were serum-starved and exposed to various concentrations (0–100 nM) of recombinant globular Adp (gAdp) and/or insulin for different time periods at 37 °C. Phosphorylation of Akt was monitored by Western blot using an antiserum against $pSer^{473}$ or $pThr^{308}$ Akt. The results demonstrate that gAdp activates Akt in dose- and time-dependent manners. When L6 cells were treated with sub-maximal concentrations of both insulin (10 nM) and gAdp (10 nM) for 10 min neither synergistic nor additive activation of Akt was observed. Similar non-synergistic or non-additive effect of gAdp on insulin-induced Akt activation was also observed in mouse C2C12 myocytes and rat vascular smooth muscle PAC cells. Moreover, pretreatment of the L6 cells with wortmannin (100 nM) for 20 min significantly reduced gAdp (100 nM) induced and insulin (100 nM) induced Akt activation by ~80 and ~70%, respectively. These data suggest that adiponectin stimulates Akt activation via the wortmannin sensitive pathway in L6 cells; and that its effects on Akt phosphorylation are not additive to those of insulin.

1. Introduction

Type 2 diabetes (T2D) is a growing public health problem characterized by an initial reduction in glucose uptake by skeletal muscle and adipose tissue due to insulin resistance caused by obesity and a complex interaction between multiple environmental and genetic factors [1–5]. Insulin resistance is compounded, later in the disease process, by a reduction in insulin secretion by pancreatic β-cells in response to increasing blood glucose [6]. Adiponectin (Adp) is a recently discovered adipokine whose levels, paradoxically, are decreased in obesity despite the increase in adipocyte mass [7–9]. Adp induces vascular smooth muscle cell differentiation [10] and also improves endothelial dysfunction elevated by FFAs level [11]. Adp suppresses triglyceride accumulation, increases fatty acid oxidation and activates the AMP kinase (AMPK) in skeletal muscles [12], improving insulin signaling [13]. Adp also suppresses glucose production and activates AMPK in liver [13]. Hence, Adp is an insulin sensitizer in skeletal muscles. Adp is involved in the regulation of whole-body energy metabolism [14]; its effects include the control of AMPK activity in skeletal muscles, liver and adipose tissues, as well as other mechanisms mediated by the control of transcription factor expression [15–17].

Akt plays a central role in cell signaling downstream of growth factors, cytokines, and other cellular stimuli. Aberrant loss or gain of Akt activation underlies insulin resistance and pathophysiological properties of T2D [18–20]. Adp binding to its receptors, AdipoR1 and AdipoR2, leads to AMPK activation, which, in turn, suppresses S6 Kinase phosphorylation of the serine residues of the insulin receptor substrate 1 (IRS-1) thereby enhancing its tyrosine phosphorylation and sensitization of insulin action [21]. Adp was also shown to stimulate the new blood vessel growth by promoting cross-talk between AMP-activated protein kinase and Akt signaling within endothelial cells [22]. It has also been reported that although gAdp alone does not promote phosphorylation of Akt it potentiates insulin induced Akt phosphorylation in C2C12 cells [23]. Recent study demonstrates a lack of AMPK involvement and implicates Akt and ERK in adiponectin signaling, leading to protection against apoptosis and stimulation of insulin gene expression and secretion in pancreatic beta cells [24]. Given the beneficial actions of Adp on insulin resistance, promotion of pharmacological strategies to restore or increase plasma adiponectin levels or adiponectin receptor expression could help reduce insulin resistance in disorders associated with obesity and T2D.

Abbreviations: gAdp, globular adiponectin; T2D, type-2 diabetes; Akt, protein kinase B; AMPK, AMP kinase; IR, insulin receptor; IRS-1, insulin receptor substrate 1; AdipoR1/R2, adiponectin receptors 1 and 2; L6, rat skeletal muscle cells; C2C12, mouse skeletal muscle cells; PAC1, rat pulmonary aorta cells; FBS, fetal bovine serum; BSA, bovine serum albumin.

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The purpose of the current study was to investigate if gAdp activates Akt independently from insulin; and if gAdp synergizes with insulin on Akt phosphorylation in the rat skeletal muscle L6 cell line.

2. Materials and methods

2.1. Materials

Minimum essential medium, Ham's eagle medium and penicillin–streptomycin were purchased from GIBCO BRL Life Technologies, Inc. Fetal bovine serum (FBS) was purchased from Hyclones and insulin-free bovine serum albumin (BSA) purchased from Fluka. Tissue culture laboratory ware was purchased from Falcon. Rat gAdp was purchased from Bio-Vision, CA. Human biosynthetic insulin was kindly supplied by Eli Lilly and Co. Antibodies to β-actin, Akt, Thr^{308} phosphorylated Akt and Ser^{473} phosphorylated Akt were purchased from Santa-Cruz or Abcam. Antibody to the active Ser^{474} phosphorylated Akt was purchased from Abcam. Electrophoretic reagents were obtained from Bio-Rad. The chemiluminescence detection reagent kit was from Perkin Elmer. All other chemicals were reagent grade and purchased from Sigma.

2.2. Cell line and cell culture

Conditions for culturing Rat L6, PAC1, and C2C12 cells were cultured essentially as described in earlier studies [25–27]. Rat L6 cells and C2C12 cells were differentiated to myotubes by culturing the cells in DMEM containing a low fetal bovine serum (2% FBS) for 5–8 days after initiation of differentiation and as described [28].

2.3. Adp and insulin stimulation

The L6 cells were cultured and differentiated in 6 well tissue culture plates. The cells were rinsed and incubated in a serum-free DMEM containing 0.1% insulin-free BSA for 8 h prior to Adp and/or insulin treatment. This step was necessary to reduce basal Akt phosphorylation. For dose response studies, Adp or insulin was added to the cells for 10 min at 37 °C, at final concentrations of 0.1, 1, 5, 10, 50 or 100 nM. Media lacking Adp and/or insulin (0 nM) served as a vehicle control. Cells were then placed on ice and rinsed twice with ice-cold phosphate buffer saline, pH 7.5 (PBS). The cells were lysed on ice in a solubilizing buffer containing 150 mM NaCl, 50 mM Tris–HCl, pH 7.4, 1% Triton X-100, 0.2% sodium deoxycholate, 0.2% sodium dodecylsulfate (SDS), 1 mM sodium ethylenediaminetetraacetate, 1 mM phenylmethylsulfonyl fluoride, 1 mM NaF, 5 μg/ml aprotinin, 5 μg/ml leupeptin, and 1 mM $NaVO_4$. Lysates were centrifuged at 10,000×*g* for 2 min and supernatants were stored at −20 °C until determination of protein concentration and Western immunoblot analysis.

2.4. Immunodetection of pAkt

Samples from control and Adp or/and insulin-treated cells were diluted with 5× sample buffer (0.5 M Tris–HCl, pH 6.8,

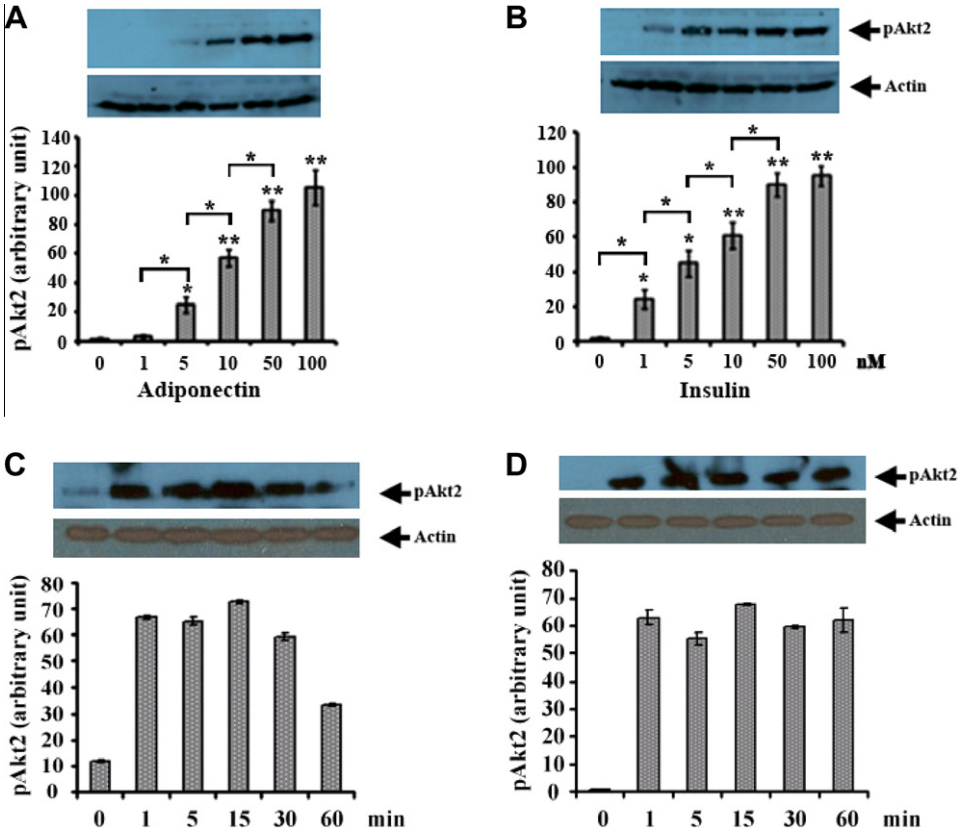

Fig. 1. Dose and time course of the effects of adiponectin and insulin on Akt phosphorylation in L6 cells. Dose-dependent adiponectin (A) and insulin (B) stimulated phosphorylation of Akt in L6 cells. The cells were serum-starved for 8 h, the medium was replaced, and the cells were incubated with the indicated concentrations of adiponectin or insulin for 10 min at 37 °C. The cells were then solubilysed and the extracts were subjected to SDS–PAGE followed by transferring to a nitrocellulose membrane and were analyzed by immunoblotting with an anti $pSer^{474}$Akt or an anti actin antisera. The arrowheads indicate the phosphorylated Akt bands or actin bands. The Western blot (upper panel) is representative of three similar experiments. The blots from 3 experiments were scanned and means ± SEM of pAkt band densities are shown (lower panel). $^{*}P < 0.05$; $^{**}P < 0.01$ vs. control (vehicle) or between the indicated groups. Time course of the effects of adiponectin (C) and insulin (D) on Akt phosphorylation in L6 cells. The cells were serum-starved for 8 h and incubated with 50 nM adiponectin or insulin for the indicated time periods at 37 °C. The Western blots were performed and analyzed as in Fig. 1A and B. Upper panels: Representative blots. Lower panels: means ± SEM of pAkt band densities calculated from three experiments.

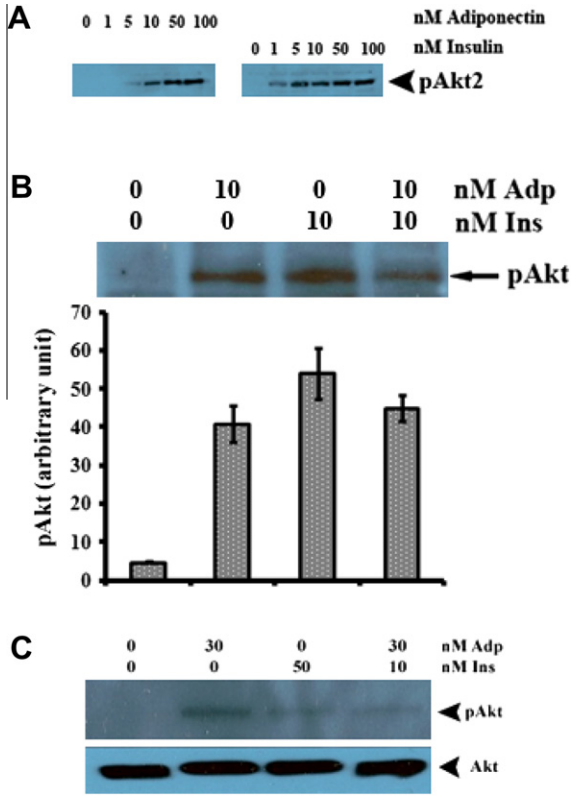

Fig. 2. Non-additive effects of adiponectin and insulin on Akt phosphorylation in rat skeletal muscle L6 and mouse skeletal muscle C2C12 cells. Adiponectin and insulin dose effects on Akt phosphorylation in L6 (A). The cells were serum-starved for 8 h and incubated with the indicated concentrations of adiponectin and insulin for 10 min at 37 °C. The cell extracts were analyzed and the blots represented same as in Fig. 1A and B. Non-additive effects of adiponectin and insulin on Akt phosphorylation in L6 (B). The cells were serum-starved for 8 h and incubated with the indicated concentrations of adiponectin ± insulin for 10 min at 37 °C. The cell extracts were analyzed as in Fig. 1A and B. Representative blots are shown. Lower panels: means ± SEM of pAkt band densities calculated from three experiments. Adiponectin stimulates phosphorylation of Ser^{474}Akt in C2C12 cells (C). The cells were serum-starved for 8 h and pretreated with vehicle or adiponectin (30 nM) for 15 min and then vehicle or insulin (10 or 50 nM) was added for 5 min at 37 °C. The cells were then solubilysed and the extracts were subjected to SDS–PAGE followed by transferring to a nitrocellulose membrane and were analyzed by immunoblotting with an anti $pSer^{474}$Akt or an anti Akt antisera. The arrowheads indicate positions of the phosphorylated Akt bands and the total Akt bands, respectively. The blot is representative of three similar experiments.

100 mM DTT, 8.5% SDS, 27.5% sucrose and 0.03% bromophenol blue) and heated to 100 °C for 3 min. Equal amounts of protein (20 μg) were resolved by 10% SDS–PAGE and transferred to nitrocellulose membranes. The active forms of Akt phosphorylated on Thr^{309} (Thr^{309} in Akt2 corresponds to Thr^{308} in Akt1 and Thr^{306} in Akt3)- or Ser ^{474} (Ser^{474} in Akt2 corresponds to Ser^{473} in Akt1 and Ser^{472} in Akt3) (p-Akt) were immunodetected using specific antisera. All primary antisera were diluted 1:1000. The secondary antisera (donkey anti-goat/goat anti-rabbit IgG, horseradish peroxidase-conjugated) were diluted 1:2000. The immunoblots were treated with chemiluminescence reagents and exposed to Amersham's hyper-film. All blots were developed and exposed under identical conditions. Autoradiograms were scanned and densitometric data were obtained using an AlphaImager. Immunoreactivity of the protein of interest detected in Western blots were quantified and compared as relative band intensities of the treated and control cells.

2.5. Statistical analysis

Data are expressed as means ± SEM. The data were analyzed by one way analysis of variance followed by the Student's *t*-tests and a *P*-value of <0.05 was considered statistically significant.

3. Results

3.1. Globular Adp activates Akt in L6 myocytes

We examined $pSer^{474}$Akt levels in L6 cells challenged with increasing concentrations of gAdp or insulin for 10 min at 37 °C. Both gAdp and insulin caused dose-dependent increase in Akt phosphorylation (Fig. 1A and B). The minimal effective concentration for gAdp was 5 nM whereas that for insulin was 1 nM. Similarly, the maximal effective concentration of gAdp was 100 nM whereas that of insulin was 50 nM.

Both gAdp and insulin increased pAkt levels in a time dependent manner (Fig. 1C and D). The effects of gAdp on pAkt peaked at 15 min (~7-fold increase above basal) and then declined gradually to ~3-fold at 60 min (Fig. 1C). On the other hand, insulin rapidly induced total cellular Akt phosphorylation, the maximum effect was reached at 1 min and the levels of pAkt remained steady for 60 min (Fig. 1D).

3.2. Akt activation by insulin and gAdp in L6 myocytes is neither synergistic nor additive

Having found that Adp activates Akt in L6 cells independently from insulin we investigated if insulin and gAdp have synergistic and/or additive effects on Akt activation. Both gAdp and insulin increased Akt phosphorylation in a dose-dependent manner. Submaximal effective concentrations of 10 nM of gAdp and insulin were selected for the additivity experiment (Fig. 2A). However, the combined effect of both insulin (10 nM) and gAdp (10 nM) was not different from the effects of individual stimulators added separately (Fig. 2B). These data demonstrate that Akt activation in L6 cells by insulin and gAdp is neither synergistic nor additive. The lack of additive effects in L6 cells suggests that insulin and gAdp stimulation of Akt phosphorylation in L6 cells occurs via a shared intracellular pathway. We also investigated if treatment of mouse muscle C2C12 cells (Fig. 2C) with Adp increases Akt phosphorylation on Ser^{474} and if the effect is synergistic or additive to that of insulin. Treating the cells with insulin (50 nM) for 5 min or with gAdp (30 nM) for 20 min increased phosphorylation of Akt in C2C12 cells, however, treating the cells with both insulin (10 nM) and gAdp (30 nM) did not result in a further increased Akt phosphorylation (Fig. 2C). These data suggest that gAdp stimulates the phosphorylation of Akt on Ser^{474} in C2C12 cells and that it does not synergize with insulin induced Akt phosphorylation in C2C12 cells.

3.3. Adp effects on Akt (Thr^{308}) activation in myocytes from rat or mouse skeletal muscle or from rat vascular smooth muscle cell lines

We also examined if gAdp stimulates the phosphorylation of Akt on Thr^{308}. We treated L6 and C2C12 myotubes, and vascular smooth muscle cells with gAdp and/or insulin. Similar to Ser^{474} phosphorylation, gAdp increased the Thr^{308} phosphorylation (Fig. 3A–C) and the effects were not additive to those of insulin when tested under sub-maximal concentration also (Fig. 3, Bottom panels, S).

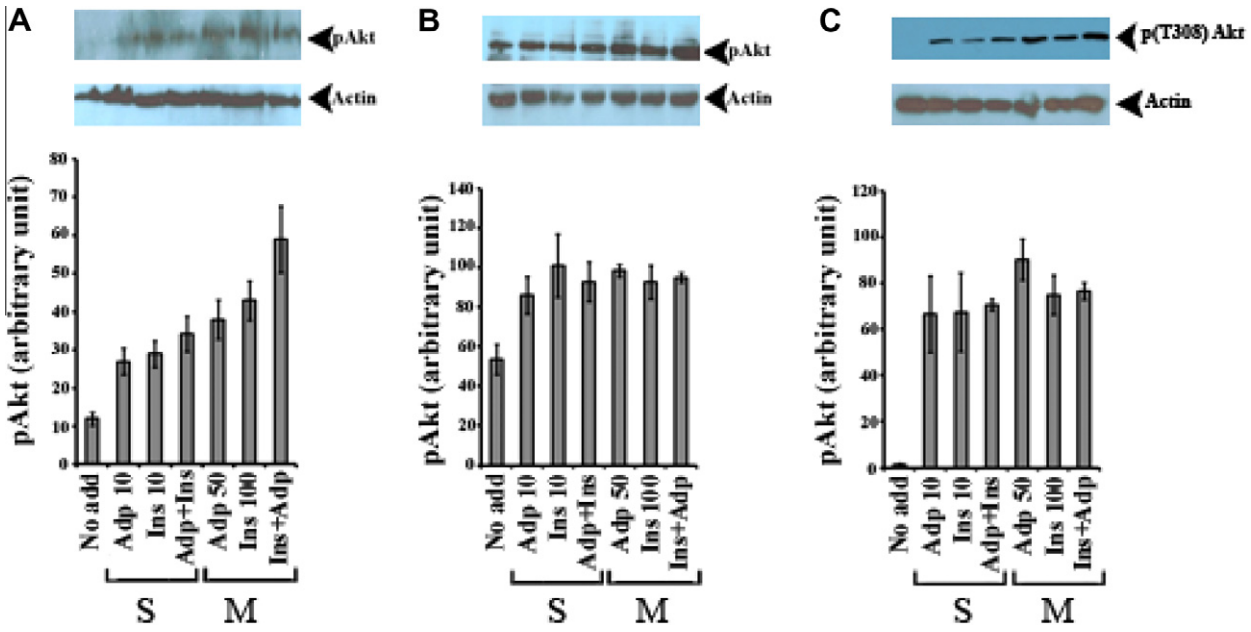

Fig. 3. Effects of adiponectin on Thr[308]Akt phosphorylation in skeletal and vascular smooth muscle cells. Serum-starved cells were pretreated with adiponectin (0, 10 or 50 nM) for 15 min and then treated with vehicle or insulin (10 or 100 nM) for 5 min at 37 °C. The rat skeletal muscle L6 (A), mouse skeletal muscle C2C12 (B), and rat vascular smooth muscle PAC (C) cells were then solubilysed and the extracts were subjected to SDS–PAGE followed by transferring to a nitrocellulose membrane and immunoblotting with an anti pThr[308]Akt or anti-actin antisera. The arrowheads indicate phosphorylated Akt bands or actin bands, respectively. Upper panels: Representative blot of 3 similar experiments. Lower panels: means ± SEM of pAkt to actin band density ratios calculated from three experiments. The letters S and M denote sub-maximal and maximal concentrations of Adp and/or insulin, respectively.

3.4. Globular Adp activates Akt via the wortmannin-sensitive pathway in L6 myocytes

Our observation that gAdp activates Akt in L6 cells independently of insulin led us to investigate if the effects of gAdp on Akt occur through the PI3 kinase pathway. We examined the effects of wortmannin, a PI3K inhibitor, on gAdp and insulin-induced Akt activation in L6 cells. Wortmannin reduced gAdp and insulin-induced Akt activation by ~80 and 70%, respectively (Fig 4). These data suggest that, similar to insulin, gAdp activates Akt via the wortmannin-sensitive pathway.

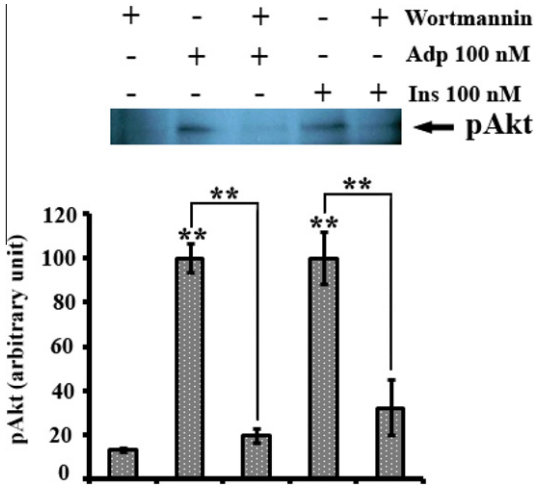

Fig. 4. Wortmannin inhibits adiponectin-induced phosphorylation of Akt in L6 myocytes. Serum-starved cells were pretreated with or without wortmannin for 20 min and then treated with either adiponectin (100 nM) or insulin (100 nM) for 10 min at 37 °C. The cells were then solubilysed and the extracts were subjected to SDS–PAGE followed by electrotransferring to a nitrocellulose membrane and were analyzed by immunoblotting with an anti pSer[474]Akt antiserum. The arrowhead indicates phosphorylated Akt bands. The Western blot shown is representative of three similar experiments. Immunoreactive p-Akt bands were scanned and expressed as relative band intensities (Bottom panels). Values are means ± SEM from three experiments. $^{*}P < 0.05$; $^{**}P < 0.01$ compared with the control or between the indicated groups.

4. Discussions

Akt plays an important role in intracellular signaling pathway regulating glucose metabolism. Adp was shown to potentiate insulin action on insulin-stimulated glucose uptake and activation of Akt. In the present study we show that globular Adp independently stimulates Akt phosphorylation on Thr[308] and Ser[474]; and that its effects on Akt are not additive to those of insulin.

Adp exists either as multimers of full-length Adp or as gAdp [9,29–31]. In this study, we used gAdp because this form has a higher affinity than full-length Adp for the AdipoR1, which is preferentially expressed in muscle cells [13]. Studies have shown that acute treatment of mice with gAdp increases fatty acid oxidation in muscle [30], and over-expression of gAdp inhibits progression of atherosclerosis in vivo [32].

The observation that gAdp stimulates the Akt phosphorylation in L6 cells in time-dependent and dose-dependent manners raises the possibility that gAdp may stimulate the same pathway stimulated by insulin. *The fact* that gAdp activation of Akt in L6 cells is neither synergistic nor additive to that of insulin suggests that adiponectin and insulin may have a similar mechanism of actions on Akt in L6 cells. These data are not consistent with what is generally known about Adp which is reported to potentiate insulin-stimulation of Akt in C2C12 cells [23]. We therefore examined C2C12 cells for gAdp stimulation of Akt phosphorylation and found that gAdp also increased the phosphorylation of Akt in C2C12 cells. Since most of the previous reports regarding Adp potentiation of insulin action on Akt phosphorylation examined Akt phosphorylation on Thr[308] we also tested the effect of gAdp

on Akt phosphorylation on Thr^{308} in L6 and C2C12 cells. Our data indicates that gAdp stimulate Akt phosphorylation on both Ser^{474} and Thr^{308} in the 3 cell lines tested, L6, C2C12 and PAC; this suggests that gAdp effects on Akt may be a general phenomenon and is not limited to one specific cell line.

Since wortmannin reduces gAdp-induced Akt activation by ~80%, gAdp activation of Akt must involve the PI3 kinase pathway. It remains to be seen although unexpected, if gAdp has any role at all on the activation of IR tyrosine kinase and/or the phosphorylation of IRS-1 before its action is transmitted through PI3 kinase to Akt activation.

In summary, in this work we provide the following evidence: (i) gAdp activates Akt in muscle cell lines; (ii) gAdp activates Akt via a wortmannin sensitive pathway; and (iii) gAdp and insulin effects on Akt activation in muscle cells are non-additive. The fact that gAdp activates Akt in 3 cell lines suggests that the effect of gAdp on Akt is a general phenomenon that is not limited to a specific cell type. These data also suggest that adiponectin and insulin may have a similar mechanism of actions on Akt.

Acknowledgments

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