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Role of ataxia telangiectasia mutated in insulin signaling of muscle-derived cell lines and mouse soleus

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Abstract

Aim—Ataxia telangiectasia mutated (ATM) reportedly plays a role in insulin-stimulated activation of Akt in some cell types but not others. The role of ATM in insulin signaling has not been firmly resolved for skeletal muscle cells, for which Akt phosphorylation is a pivotal step in stimulation of glucose transport. Accordingly, our aim was to determine the role of ATM in insulin effects for cell lines derived from skeletal muscle and for skeletal muscle. **Methods:** We examined insulin effects in L6 myotubes, mouse soleus, C2C12 myotubes, and differentiated rhabdomyosarcoma (RD) cells in the presence and absence of a low concentration (1 μ M) of the ATM inhibitor KU55933. We also compared insulin signaling in C2C12 cells expressing shRNA against ATM and control cell lines (empty vector; cells expressing non-targeting shRNA).

Results—In L6 myotubes and mouse soleus muscle, KU55933 inhibited insulin-stimulated phosphorylation of the 160 kDa substrate of Akt (AS160) despite no effect on Akt. In contrast, KU55933 prevented insulin-stimulated Akt phosphorylation in C2C12 myotubes. Furthermore, C2C12 myotubes expressing shRNA against ATM displayed reduced insulin-stimulated Akt phosphorylation compared to controls. KU55933 also decreased insulin-stimulated Akt phosphorylation in differentiated RD cells.

Conclusion—These model-dependent differences in the role of ATM in insulin action demonstrate a role of ATM in insulin-stimulated phosphorylation of Akt (in C2C12 and RD cells) but also allow the elucidation of a novel, Akt-independent role of ATM (in L6 myotubes and mouse soleus, at the level of AS160) in insulin signaling.

Keywords

ATM; Akt; atypical PKC; AS160; IRS-1; skeletal muscle; myotubes

Introduction

Skeletal muscle plays a prominent role in blood glucose homeostasis. For example, most of the glucose cleared from the blood in response to insulin is stored in skeletal muscle (Jue et al. 1989; Shulman et al. 1995). Insulin-stimulated glucose transport in skeletal muscle is

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dependent on a signaling cascade involving, among other factors, tyrosine phosphorylation of the insulin receptor and insulin receptor substrate (IRS) proteins, activation of phosphatidylinositol 3-kinase (PI3K), phosphorylation of Akt, and phosphorylation of the Akt substrate of 160 kDa (AS160) (Krook et al. 2004).

Ataxia telangiectasia mutated (ATM), a serine-threonine kinase of the phosphatidylinositol-3 kinase (PI3K)-related protein kinase family, plays well-characterized nuclear roles in cell cycle control and responses to DNA damage (Lavin 2008). However, ATM has also been shown to participate in insulin action, such as in insulin-stimulated phosphorylation of the eIF-4E-binding protein 1 (4E-BP1) in HEK 293T cells (Yang & Kastan 2000) and insulin-stimulated activation of Akt in Cos7 cells (Viniestra et al. 2005). Consistent with this, mice that are homozygous for a truncation mutation that prevents ATM activity are hyperglycemic compared to wild-type mice during oral glucose tolerance tests (Miles et al. 2007). Mice that are heterozygous for the ATM deficiency are also hyperglycemic during oral glucose tolerance tests (Schneider et al. 2006). On the other hand, Hresko et al have shown that ATM plays no role in insulin-stimulated activation of Akt in cultured adipocytes (Hresko & Mueckler 2005).

It is clear that ATM plays a role in insulin-stimulated glucose transport in myotubes. For example, transfection of a kinase-dead construct of ATM into L6 myoblasts prevented insulin-stimulated GLUT4 translocation, while GLUT4 translocation was unaffected by transfection of myoblasts with the wild-type form of ATM. Halaby et al (Halaby et al. 2008) showed that 10 μ M KU55933 (an ATM inhibitor) prevented insulin-stimulated Akt phosphorylation and insulin-stimulated glucose transport in L6 myoblasts. However, at a concentration of 10 μ M, KU55933 (which has an IC_{50} of 16.6 μ M for inhibition of PI3K (Hickson et al. 2004)) could possibly have interfered with insulin action by inhibition of PI3K (not necessarily by inhibition of ATM). Thus, while it is attractive to speculate that ATM acts upstream of Akt in insulin action, this has not been firmly established in myotubes. Given that there are likely to be cell-line specific differences in the role of ATM in insulin-stimulated phosphorylation of Akt (i.e. ATM is either necessary (Viniestra et al. 2005) or non-essential (Hresko & Mueckler 2005)), the issue of the role of ATM in insulin signaling still needs to be resolved for skeletal muscle cells.

An aim of the current project was to use inhibitor and shRNA approaches to determine whether ATM plays a role in insulin-stimulated Akt phosphorylation. In the process, we found cell-line differences in the role of ATM in insulin-stimulated Akt phosphorylation which allowed us to uncover a novel role of ATM in regulation of insulin signaling downstream of Akt, at the level of the Akt Substrate of 160 kDa (AS160).

Materials and Methods

Materials

Dulbecco's Modified Eagle's Medium (DMEM) was purchased from Washington University School of Medicine Tissue Culture Support Center (St. Louis, MO). FetalPlex Animal Serum Complex and antibiotic/antimycotic solution (10,000 U penicillin/ml, 10 mg streptomycin/ml, 0.025 mg fungizone/ml) were obtained from Gemini Bio-Products (Woodland, CA). Trypsin / EDTA and phosphate-buffered saline (PBS) were from Washington University School of Medicine Tissue Culture Support Center (St. Louis, MO). Insulin was from Eli Lilly & Co. (Indianapolis, IN). 3 H-labeled 2-deoxyglucose (2-DG) was purchased from American Radiolabeled Chemicals (St. Louis, MO). Cytochalasin B was from Sigma Chemical Chemical Co. (St. Louis, MO). 2-Morpholin-4-yl-6-thianthren-1-yl-pyran-4-one (KU55933) was a generous gift from Dr. Graeme Smith (KuDOS Pharmaceuticals LTD, Cambridge, UK). Horseradish peroxidase-conjugated goat anti-rabbit IgG and horseradish peroxidase-

conjugated goat anti-mouse IgG were from Pierce Biotechnology (Rockford, IL). Antibodies against phosphorylated Akt (P-Akt) S473 and T308, Akt, phosphorylated insulin receptor (P-IR), IR, IRS-1, GAPDH, and P-PKC ζ were obtained from Cell Signaling Technology (Beverly, MA). A phospho-IRS-1 Y612 antibody was from Novus Biologicals (Littleton, CO). The antibody against ATM was obtained from Sigma-Aldrich Chemical Co.

Cell culture

L6 (rat-derived) and C2C12 (mouse-derived) myoblasts and human rhabdomyosarcoma (RD) cells were obtained from the American Type Culture Collection (Rockville, MD). Cell lines were maintained in a humidified incubator at 37°C with 5% CO₂. Cells were cultured in 100-mm polystyrene culture dishes in DMEM supplemented with 10% FetalPlex (Gemini Bio-Products, and 1% of a 100× antibiotic antimycotic mixture (Gemini) and were passaged every two days as previously described (Somwar et al. 2001; Smith J.L. et al. 2005a; Smith J.L. et al. 2005b; Ju et al. 2007). For experiments, cells were plated in 24-well plates (for glucose transport assays) or 6- or 12-well plates (for western blot analyses), allowed to recover overnight in growth medium, and then supplied with differentiation medium (DMEM containing 2% horse serum and 1% antibiotic antimycotic mixture). Differentiation medium was changed every 48 h. Experiments were initiated when cells appeared by visual inspection to be differentiated (usually 2-4 days for C2C12 myotubes, 4-7 days for L6 cells, and 4 days for RD cells). Cells were serum-starved for 3 h (in DMEM with antimycotic/antibiotic, but no serum) before experiments.

Insulin action after treatment with KU55933, an ATM inhibitor

To determine whether ATM might be involved in regulation of insulin signaling and glucose transport in muscle cells, we relied on the use of a specific ATM inhibitor, KU55933 (a gift of Kudos Pharmaceuticals, Cambridge, UK). KU55933 inhibits ATM with an IC₅₀ (12.9 nM) that is more than 1000-fold lower than the IC₅₀ for PI3K (Hickson et al. 2004). Compared to the IC₅₀ for KU55933 against ATM, the IC₅₀s for the PI3K-related kinases mTOR, DNA protein kinase, and ATR are higher by ~700-fold, ~200-fold, and ~8,000-fold, respectively. However, at a concentration at which it is commonly used (10 μM), KU55933 could potentially also inhibit PI3K, which has an IC₅₀ of 16.6 μM for inhibition of PI3K (Hickson et al. 2004). Thus, in our studies of the role of ATM in insulin signaling, we used 1 μM KU55933 to avoid potential inhibition of PI3K.

Myotubes were serum-starved for 3 h, incubated for 30 min with 1 μM KU55933 (in DMSO vehicle) or DMSO (0.1% final concentration of DMSO in medium), then exposed to 0 or 100 nM insulin (with KU55933 and/or DMSO present if they had been present in the previous step) for 20 min before cells were harvested. For cell harvests, plates were placed on ice, media was aspirated, and wells were quickly washed three times with HBS or phosphate-buffered saline. Cells were scraped in ice-cold buffer containing phosphatase and protease inhibitors (50 mM HEPES, pH 7.4, 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1 mM EDTA, 1.5 mM MgCl₂, 10 mM sodium pyrophosphate, 100 mM NaF, 2 mM Na₃VO₄, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 0.5 μg/ml pepstatin, and 0.2 mM PMSF). Lysates were centrifuged at 14,000×g for 10 min, and the supernatant was taken. The concentration of soluble protein was measured by the BCA method. Western blot analysis was performed as described below.

For glucose transport assays, cells were serum-starved for 3 h before incubation in serum-free medium for 30 min in the presence or absence of 1 μM KU55933. After the preliminary incubation with vehicle or KU55933, the myotubes were incubated for 20 min with 0 nM or 100 nM insulin, with DMSO or KU55933 still present. Myotubes were rinsed with glucose-free HEPES-buffered saline (HBS: 20 mM HEPES, pH 7.4, 140 mM NaCl, 5 mM KCl, 2.5 mM MgSO₄, 1 mM CaCl₂, pH 7.4) and then assayed for rates of 2-deoxyglucose (2DG) uptake

as previously described (Somwar et al. 2001; Smith J.L. et al. 2005a; Ju et al. 2007). Glucose transport assays were performed by incubating cells with glucose-free HBS containing radiolabeled 2-DG (3 μ Ci/ml), 10 μ M unlabeled 2-DG, and insulin, KU55933, or DMSO (if they had been present in the previous step) for 10 min at room temperature. Other cells were incubated with 10 μ M cytochalasin B (which blocks GLUT-mediated glucose uptake) before glucose transport assays to determine non-specific 2DG uptake, which was subtracted from 2DG transport rates for other cells. After the 10 min incubation, the transport medium was aspirated, and the cells were rinsed twice with ice-cold 0.9% saline to stop 2DG uptake. Following the termination of 2DG uptake, cells were lysed in 0.2 N NaOH containing 0.2% SDS. The protein concentrations of cell lysates were determined by the bicinchoninic acid (BCA) assay method (Pierce, Rockford, IL), and 3 H in samples was assessed with a microplate scintillation counter.

Incubation of mouse skeletal muscle

Mice (129S6/SvEvTac, The Jackson Laboratory, Bar Harbour, ME) were bred and housed in an AALAC approved animal facility with free access to food and water. Male mice (29.4 $\pm$ 1.5 g) were anesthetized with sodium pentobarbital (50 mg/kg), and soleus muscles (including tendons) were removed and incubated in vitro generally as previously described (Fisher et al. 2002). Muscles were placed in incubation medium, which consisted of Krebs Henseleit bicarbonate buffer containing 8 mM glucose, 32 mM mannitol, and 0.1% radioimmunoassay grade bovine serum albumin. Incubation vials were kept at 35 °C with gentle shaking and gassed with 95% O₂:5% CO₂. After an hour of recovery after dissection, muscles were placed in incubation medium containing 0.1% dimethyl sulfoxide (DMSO, vehicle) or 1 μ M KU55933 for 30 min. Then, muscles were placed in medium (containing DMSO or KU55933, if they had been present in the previous step) in the absence or presence of 2 mU/ml insulin for 30 min. Muscles were blotted, trimmed, and instantly frozen with clamps that were pre-cooled in liquid nitrogen. Muscles were homogenized in Kontes ground glass tubes in the lysis buffer described above for cell harvests, and BCA protein assays were performed.

All animal procedures were approved by the Saint Louis University Institutional Animal Care and Use Committee.

Creation of ATM knockdown C2C12 cell lines by shRNA lentiviral transduction

In order to corroborate findings obtained through the use of the ATM inhibitor, a cell line expressing shRNA against ATM was produced using a lentiviral vector that contained a puromycin resistance cassette. C2C12 myoblasts were transduced at an MOI of 5 with the lentiviral particles which express the shRNA targeting ATM (Sigma TRCN0000012646, CCGGCGATGGAAGTTATGCGGAGTTCTCGAGAACTCCGCATAACTTCCATCGTT TTT). Cells were transduced in parallel with control lentiviral particles containing no shRNA insert (MISSION pLKO.1-puro, Sigma SHC001V) for expression of puromycin resistance only and with non-targeting shRNA control particles (Sigma SHC002V, CCGGCAACAAGATGAAGAGCACCAACTCGAGTTGGTGCTCTTCATCTTGTTG TTTTT). Cell lines were maintained for several passages in DMEM containing 10% fetal bovine serum, 4 mM L-glutamine, 1 mM sodium pyruvate, and 2 μ g/ml puromycin. ATM expression in myoblasts (from empty vector, non-targeting shRNA, and ATM shRNA lines) was determined by QuantiGene Assay (QuantiGene Discover Assay Kit from Panomics) using QuantiGene 1.0 Probe Set (Mouse Atm). The results from three biological replicates of each cell line show that the RNA expression level of ATM in ATM shRNA cells is about 75% less than control (Empty vector and Non-targeting shRNA) cells.

For experiments, cells were multiplied, grown, and maintained in the growth and differentiation media described above in the general section on cell culture (i.e. puromycin-free medium).

Myotubes from the three cell lines (empty vector, non-targeting shRNA, and ATM shRNA) were serum-starved for 3 h, then incubated in either 0 nM or 100 nM insulin for 30 min before harvest and western blot analysis, as described above. Samples were probed for ATM, P-ATM S1981 (an autophosphorylation site on ATM), P-Akt S473 and T308, and Akt.

RD cells

Given the contrasting reports regarding the requirement for ATM in insulin-stimulated Akt phosphorylation (Viniegra et al. 2005; Hresko & Mueckler 2005), we examined the effect of ATM inhibition on stimulation of Akt phosphorylation in an additional cell culture model for skeletal muscle: human rhabdomyosarcoma (RD) cells, a line derived from skeletal muscle that differentiates into multinucleated, myotube-like cells. Differentiated, serum-starved cells were incubated for 30 min in medium containing vehicle (DMSO) or 1 μ M KU55933, then incubated an additional 20 min in the absence or presence of 1 μ M insulin before processing for glucose transport assays or western blots.

Western blot analysis

Samples were solubilized in Laemmli sample buffer containing dithiothreitol and boiled for 5 min, and samples were subjected to SDS-PAGE as previously described (Ju et al. 2007). For detection of ATM (~350 kDa), we used a system designed for separation of large proteins: precast 3-8% Tris-Acetate gels (Invitrogen) and HiMark (Invitrogen) molecular weight markers (31 kDa-460 kDa). Proteins were transferred to nitrocellulose membranes, and then the membranes were blocked in Tris-buffered saline containing 0.1% Tween and 5% non-fat dry milk. Proteins were detected by incubation of blots with primary antibodies, incubation with horseradish peroxidase conjugated anti-rabbit or mouse IgG, and then visualization by chemiluminescence (Western Lightning; PerkinElmer, Waltham, MA). For determination of AS160 phosphorylation, membranes were probed with AS160 antibodies (Cell Signaling Technologies) or antibodies against phosphorylated Akt substrates (P-AS, Cell Signaling Technologies). The P-AS band corresponding to the migration of AS160 phosphorylated AS160.

Statistical analysis

Data were generally analyzed by 2×2 ANOVA, followed by post-hoc LSD comparisons if an interaction was found. In some instances, data were analyzed by one-way ANOVA with post-hoc comparisons when appropriate. A level of $P < 0.05$ was regarded as significant.

Results

Insulin action in L6 myotubes

Inhibition of ATM did not affect phosphorylation of Akt in L6 myotubes stimulated with 100 nM insulin (fig 1A, quantitation in table 1). To rule out the possibility that ATM's role in L6 myotubes might be to increase insulin sensitivity--as opposed to maximum responsiveness--in regard to Akt phosphorylation, we repeated the experiment using a lower insulin concentration. The ATM inhibitor did not decrease Akt phosphorylation stimulated by 25 nM insulin (fig 1B, quantitation in table 1). However, despite no effect on Akt phosphorylation, the ATM inhibitor reduced glucose transport in the L6 myotubes (fig 1C). This suggests that ATM may have some action independent of or downstream of Akt that is important in stimulation of glucose transport. Accordingly, we assessed phosphorylation of Akt substrate of 160 kDa (AS160) in response to insulin in L6 myotubes. As shown in fig 1D and 1E, insulin-stimulated phosphorylation of AS160 at Akt target sites (P-AS) was reduced by the ATM inhibitor. The effect of the ATM inhibitor to reduce AS160 phosphorylation was unlikely to have been caused through inhibition of ATM-related kinases that participate in classical insulin signaling (e.g.

PI3K and mTOR), as insulin-stimulated Akt phosphorylation (which is dependent on PI3K activity and action of mTOR-ricor on Akt S473) was unaffected by 1 μ M KU55933. We have found that 1 μ M KU55933 reduces phosphorylation of p53 at its ATM target site in L6 myotubes (data not shown), demonstrating that this concentration of KU55933 is likely to be sufficient to inhibit ATM.

Insulin signaling in mouse soleus

We investigated the effect of inhibition of ATM on insulin signaling in isolated mouse soleus. As shown in figure 2 (A, D, and E), insulin-stimulated Akt phosphorylation was not affected by the presence of the ATM inhibitor KU55933. However, as for L6 myotubes, inhibition of ATM blunted insulin-stimulated phosphorylation of AS160 (figure 2B,F; $P=0.05$).

We then investigated the possibility that inhibition of ATM might interfere with phosphorylation of atypical PKC ζ/λ (aPKC), and that reduced aPKC phosphorylation would correlate with reduced AS160 phosphorylation. As shown in figures 2C and 2G, insulin stimulated phosphorylation of aPKC was quite variable. However, basal and insulin-stimulated phosphorylation of aPKC were not affected by the presence of the ATM inhibitor.

Insulin action in C2C12 myotubes

We performed a dose-response curve for inhibition of insulin-stimulated glucose transport by KU55933. As shown in figure 3, 1 μ M KU55933 completely prevented insulin-stimulated glucose transport in C2C12 myotubes without interfering with basal glucose transport. In contrast, 10 μ M KU55933 reduced basal glucose transport by about 75% (data not shown). Thus, we selected 1 μ M KU55933 as the concentration to use for subsequent experiments with C2C12 myotubes. 1 μ M KU55933 prevented insulin-stimulated S473 and T308 phosphorylation of Akt ($P < 0.05$, fig 4A-C). In contrast to findings for Akt, the ATM inhibitor did not negatively affect insulin-stimulated phosphorylation of the insulin receptor and did not statistically significantly depress Y612 phosphorylation of IRS-1.

To verify results obtained with the use of the ATM inhibitor, we used C2C12 cell lines transduced with lentivirus clones containing shRNAs against ATM and negative controls, including empty lentivirus and lentivirus containing a non-targeting shRNA. ATM bands ran where expected (between 263 kDa and 460 kDa), were the only bands present at the expected molecular weight (~350 kDa), and were substantially reduced in intensity in cells expressing shRNA targeting ATM. Thus, the antibodies appear suitable for specific detection of ATM. As shown in figure 5A, cells expressing the shRNA against ATM expressed ~75% less ATM protein than the negative control cells. Insulin stimulated Akt S473 and T308 phosphorylation (fig 5) in control cell lines but not in cells expressing the shRNA targeting ATM.

In summary, both a specific inhibitor of ATM (fig 4) and shRNA against ATM (fig 5) interfere with stimulation of Akt phosphorylation by insulin in C2C12 myotubes.

Role of ATM in Akt phosphorylation in RD cells

Our findings regarding a role for ATM in insulin action in C2C12 (mouse) cells mirror the previously-reported of a requirement for ATM for full activation of Akt by insulin in Cos7 cells (Viniegra et al. 2005) but do not agree with a report that insulin-stimulated Akt phosphorylation is normal in NIH 3T3-L1 adipocytes treated with siRNAs against ATM (Hresko & Mueckler 2005) or with our data from L6 myotubes and mouse soleus. Because of these discrepant findings, we assessed whether inhibition of ATM would impair insulin action in an additional cell culture model for skeletal muscle: differentiated RD (human) cells. As shown in fig 6, 1 μ M KU55933 reduced insulin-stimulated Akt phosphorylation in

differentiated RD cells. Thus, human (fig 6) and mouse (fig 4-5) cells derived from skeletal muscle display a similar reliance on ATM in insulin-stimulated Akt phosphorylation.

Discussion

The new information provided by the study is that cell-line-dependent differences in the role of ATM in insulin signaling provide support for specific experimental models in which to study the role of ATM in insulin-stimulated activation of Akt (C2C12 and RD cells) and to examine Akt-independent effects of ATM (L6 cells and mouse skeletal muscle) in mediation of insulin's effects.

Much work in the field has been focused on identifying a point of convergence of insulin-dependent and insulin-independent signaling leading to glucose transport. At present, a pair of GTPase activating proteins, AS160 and TBC1D1, have been implicated as signaling nodes downstream of both Akt and insulin-independent signaling leading to glucose transport (Kramer et al. 2006; Thong et al. 2007; Taylor et al. 2008). The current work is noteworthy in that it elucidates roles of ATM upstream of Akt (at least for cell lines derived from mouse and human skeletal muscle) and downstream of Akt (for rat L6 myotubes). Our data from L6 myotubes suggest that ATM may play some role in regulation of AS160 phosphorylation that does not involve a direct effect on Akt itself.

Yang and Kastan (Yang & Kastan 2000) demonstrated that ATM participates in regulation of protein translation by insulin. Consistent with this, it has been found that ATM overexpression and expression of the PI3K domain of ATM are sufficient to increase Akt S473 phosphorylation in Cos7 (monkey fibroblast-like) cells (Viniegra et al. 2005). Conversely, RNAi-mediated decreases in ATM expression prevented insulin-stimulated phosphorylation of Akt S473 in the Cos7 cells (Viniegra et al. 2005). Likewise, ATM-deficient human and mouse fibroblasts exhibited severely impaired insulin-stimulated Akt S473 phosphorylation compared to fibroblasts with normal ATM expression (Viniegra et al. 2005). In contrast, Hresko et al have shown that siRNA-mediated reduction in ATM expression has no effect on insulin-stimulated Akt phosphorylation in 3T3-L1 adipocytes (Hresko & Mueckler 2005).

Halaby et al (Halaby et al. 2008) showed that 10 μ M KU55933 (the ATM inhibitor used in the current study) prevented insulin-stimulated Akt phosphorylation and insulin-stimulated glucose transport in L6 myoblasts. In contrast, in the current study, we have found that 1 μ M KU55933 (a concentration we chose to avoid potential inhibition of PI3K), interferes with insulin-stimulated glucose transport but does not affect insulin-stimulated Akt phosphorylation. The differences between the findings in the current study and the previous report (Halaby et al. 2008) could have been a result of use of myotubes (the current study) instead of myoblasts (Halaby et al. 2008) or from differences in the KU55933 concentrations (1 μ M vs. 10 μ M). Regardless, in our system, the ATM inhibitor has no effect on insulin-stimulated Akt phosphorylation in L6 myotubes. The reasons for the cell-line differences among C2C12, RD, and L6 cells are unclear, as are the reasons for the contrasting reports regarding a role (or no role) of ATM in insulin-stimulated activation of Akt in other cell types (Viniegra et al. 2005; Hresko & Mueckler 2005). Nevertheless, the lack of influence of ATM inhibition on insulin-stimulated Akt phosphorylation in L6 myotubes allowed us to uncover an effect of ATM inhibition downstream of Akt, at AS160.

The effect of ATM inhibition on insulin signaling was qualitatively similar in L6 myotubes and mouse soleus (i.e. effects at AS160 but not Akt). Thus, it appears that differences in effects of ATM inhibition among the various models presented in this paper are not species specific. It is interesting to note that inhibition of ATM appears to affect insulin-stimulated Akt phosphorylation in cells (e.g. C2C12 cells) that are not considered good cell culture models

for insulin action (Kotliar & Pilch 1992; Tortorella & Pilch 2002) or that require high levels of insulin to stimulate insulin signaling (e.g. we needed a 10-fold higher insulin concentration in RD cells than in L6 cells to stimulate Akt phosphorylation). In contrast, inhibition of ATM did not affect insulin-stimulated Akt phosphorylation in models considered to be more insulin responsive and/or insulin sensitive (e.g. mouse muscle, L6 myotubes, and 3T3L1 adipocytes (Hresko & Mueckler 2005)) than C2C12 cells.

Atypical PKC (aPKC) isoforms have been shown to be activated by insulin (Beeson et al. 2003; Sajan et al. 2006; Farese et al. 2007; Temofonte et al. 2009) in L6 myotubes, 3T3-L1 adipocytes, and mouse and human skeletal muscle and to be necessary to insulin-stimulated glucose transport in L6 myotubes, adipocytes, and skeletal muscle (Sajan et al. 2006; Farese et al. 2007). While the major aPKC in L6 cells is PKC ζ and the major isoform in 3T3-L1 adipocytes and skeletal muscle is PKC λ , the isoforms seem functionally interchangeable. For example, impairment of insulin-stimulated glucose transport by RNAi-mediated knockdown of the major aPKC in L6 cells and 3T3-L1 adipocytes is rescued by expression of the other isoform (i.e. PKC λ in L6 cells and PKC ζ in adipocytes). The possibility that aPKCs stimulate glucose transport by phosphorylation of AS160 has not been investigated, though activation of convention/novel PKC forms has been reported to increase AS160 phosphorylation (Thong et al. 2007). In the current study, phosphorylation of aPKC (which is necessary to aPKC activation) was not altered by ATM inhibition in mouse soleus, so it appears that the reduction in insulin-stimulated AS160 phosphorylation in the presence of an ATM inhibitor occurs without reductions in phosphorylation of either Akt or aPKC. Several participants in insulin signaling, including IRS-2, Akt3, protein phosphatase 2A, Vamp2, and Synip, have been identified as ATM substrates (Matsuoka et al. 2007). It is possible that these or other players in insulin signaling that have yet to be identified as ATM substrates could mediate ATM's actions on insulin signaling.

Cases of extreme insulin resistance for patients with ataxia telangiectasia (A-T) have been reported (Schalch et al. 1970; Blevins, Jr. & Gebhart 1996). About 5% of the 340 patients with A-T in one registry were diagnosed with diabetes (Swift et al. 1993), which is an unusually high rate, given the relatively young age of A-T patients. Consistent with these reports of insulin resistance in patients with A-T, IRS-2 associated PI3K activity and Akt S473 phosphorylation after a hyperinsulinemic euglycemic clamp were reportedly lower in liver of ATM +/- mice (on an Apo E -/- background) compared to ATM +/+ mice on the same background (Schneider et al. 2006). In aorta, phosphorylation of IRS-1 S307 (a JNK target site associated with insulin resistance when it is phosphorylated) and JNK phosphorylation were greater in ATM mutants (ATM +/- and -/-) compared to wild type animals (Schneider et al. 2006). Glucose tolerance was decreased in ATM +/- mice (compared to ATM +/+ mice) on Apo E -/-, LDLR null, and C57BL/6 backgrounds, and heterozygosity for the ATM mutation (compared to ATM +/+ animals) reduced the decline in blood glucose concentrations during insulin tolerance tests for animals on Apo E -/- and LDLR null backgrounds. Furthermore, while ATM -/- mice might have impaired insulin secretion, they also are hypoglycemic compared to wild-type mice during oral glucose tolerance tests even at time points for which insulin concentrations do not differ between ATM -/- and wild-type mice (Miles et al. 2007). Together, reports regarding humans and mice suggest that ATM plays some role in insulin signaling and insulin-stimulated glucose disposal.

In summary, while we have found some cell line differences for the role of ATM in insulin action (i.e. ATM activity plays a role in insulin-stimulated Akt phosphorylation cell lines derived from mouse and human skeletal muscle, but not in rat L6 myotubes or mouse soleus), these differences have helped in the elucidation of a novel role for ATM in insulin signaling, downstream of Akt. For example, inhibition of ATM reduces glucose transport in L6 myotubes, despite normal insulin-stimulated Akt phosphorylation. Thus, we have uncovered experimental

systems (L6 myotubes and mouse skeletal muscle) for further investigation of the potential role of ATM in insulin effects downstream of Akt.

Acknowledgments

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Figure 1. Effects of ATM inhibition on insulin action in L6 myotubes

L6 myotubes were serum starved, incubated with 1 μ M KU55933 or vehicle (DMSO) for 30 min, and subsequently exposed to 0, 25 nM, or 100 nM insulin for 20 min before western blots or glucose transport assays. A and B: representative western blots for P-Akt S473, P-Akt T308, and Akt after cells were exposed to the indicated concentrations of insulin. Quantitation of P-Akt/Akt is shown in Table 1. C: insulin-stimulated glucose transport in the absence or presence of the ATM inhibitor. * $P < 0.001$ (group with treated with insulin+KU55933 vs. group treated with insulin+vehicle), $n = 20$ -24/group. D: representative western blots for phosphorylated Akt substrate (P-AS) corresponding to AS160 for myotubes treated in the absence or presence of KU55933 and the absence or presence of 100 nM insulin. E: quantitation of P-AS/AS160 ($n = 12$ /group; † $P < 0.05$ vs. all other groups. Data are means with SEs.

mouse soleus

Figure 2. Effects of ATM inhibition on mouse soleus

Isolated soleus muscles (n=4/groups) were incubated in the absence or presence of the ATM inhibitor KU55933 and in the absence or presence of 2 mU/ml insulin. A, C, D: representative blots of phosphorylated Akt (P-Akt) and quantitation. *P<0.01 vs. corresponding control without insulin. B, E: representative blots of phosphorylated Akt substrate (P-AS) corresponding to AS160 and quantitation. †P≤0.05 vs. all other groups. C, F: representative western blot of phosphorylated atypical PKC ζ/λ (T410/403) and quantitation. Bars denote means with SEs.

Figure 3. Dose-response curve for effects of KU55933 (an ATM inhibitor) on insulin-stimulated glucose transport in C2C12 myotubes

C2C12 myotubes were incubated with vehicle (DMSO) or inhibitor for 30 min, then incubated for 20 min with the continued presence of vehicle or inhibitor but also in the absence or presence of 100 nM insulin before 2-deoxyglucose (2DG) transport assays. $n=8-10/\text{group}$. * $P<0.05$ vs. corresponding control without insulin, † $P<0.05$ vs. insulin control without KU55933.

Figure 4. KU55933 diminishes insulin-stimulated Akt phosphorylation in C2C12 myotubes
 C2C12 myotubes were serum starved for 3 h, and they were exposed to DMSO or 1 μ M KU55933 for 20 min, followed by another 20 min incubation in the presence or absence of 100 nM insulin. A: representative P-Akt S473, P-Akt T308, and Akt Western blots. B and C: densitometry of phosphorylation of Akt Ser473 and Akt Thr308 bands. Values are means $\pm$ SE. Insulin $\times$ KU55933 interaction ($P < 0.05$). *Significantly different from other groups, $P < 0.05$; $n = 4$ / group. D and E: representative western blots and quantitation of P-IR/IGFR and IR. Main effect for insulin ($P < 0.01$), $n = 4$ / group. F and G: representative western blots and quantitation of P-IRS1 and IRS1. Main effect for insulin ($P < 0.01$) and tendency toward insulin $\times$ KU55933 interaction ($P = 0.11$), $n = 8$ / group.

Figure 5. Insulin-stimulated Akt phosphorylation is decreased in C2C12 myotubes expressing shRNA targeting ATM

Cell lines created by lentiviral infection of C2C12 cells (empty vector, non-targeting shRNA, and shRNA against ATM) were differentiated into myotubes, serum-starved, and incubated in the absence or presence of 100 nM insulin. A: representative western blots of ATM, P-Akt S473, P-Akt T308, and Akt. B: Quantitation of P-Akt S473, C: Quantitation of P-Akt T308.

* $P < 0.05$, greater than corresponding group without insulin. † $P < 0.05$, lower than insulin-stimulated empty vector and non-targeting shRNA lines. $n = 3-4$ /group. Data are means $\pm$ SEs.

Figure 6. Inhibition of ATM prevents insulin-stimulated Akt phosphorylation in RD cells
 RD cells were differentiated, serum-starved, and incubated with 1 μ M insulin. A: representative western blots of P-Akt S473, P-Akt T308, and Akt. B: Quantitation of P-Akt S473 C: Quantitation of P-Akt T308. For both panels A and B, there was a significant ($P < 0.05$) inhibitor $\times$ insulin interaction. * $P < 0.05$, greater than vehicle (DMSO) group. † $P < 0.05$, lower than group with insulin but without ATM inhibitor. $n = 3/\text{group}$. Data are means $\pm$ SEs.

No impact of ATM inhibition on insulin-stimulated phosphorylation of Akt in L6 myotubes

Table 1

Myotubes were incubated for 30 min with DMSO (vehicle) or 1 μ M KU55933 and then with the indicated concentration of insulin for 20 min (with the continued presence of DMSO or KU55933). Representative western blots for these data are shown in fig 5. Quantitation of phosphorylated protein was normalized to quantitation of total protein (e.g. P-Akt/Akt). Values are in relative units, represented by means $\pm$ SEs.

	DMSO	KU55933	DMSO + INSULIN	KU55933 + INSULIN	n	main effect for insulin
100 nM insulin						
P-Akt(S473)/Akt	1.0 $\pm$ 0.2	1.0 $\pm$ 0.3	6.4 $\pm$ 0.5	7.0 $\pm$ 0.7	4/group	P<0.001
P-Akt(T308)/Akt	1.0 $\pm$ 0.1	0.8 $\pm$ 0.2	5.1 $\pm$ 0.3	5.4 $\pm$ 0.3	4/group	P<0.001
25 nM insulin						
P-Akt(S473)/Akt	1.0 $\pm$ 0.2	1.1 $\pm$ 0.3	2.4 $\pm$ 0.3	2.2 $\pm$ 0.3	10/group	P<0.001
P-Akt(T308)/Akt	1.0 $\pm$ 0.2	1.3 $\pm$ 0.2	1.9 $\pm$ 0.1	1.7 $\pm$ 0.2	10/group	P<0.001