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Impaired insulin-stimulated glucose transport in ATM-deficient mouse skeletal muscle

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Abstract

There are reports that ataxia telangiectasia mutated (ATM) plays a role in insulin-stimulated Akt phosphorylation, though in some cell types this is not the case. As Akt plays a key role in insulin signaling leading to glucose transport in skeletal muscle, the predominant tissue in insulin-stimulated glucose disposal, we examined whether insulin-stimulated Akt phosphorylation and/or glucose transport would be decreased in skeletal muscle of mice lacking functional ATM compared to muscle from wild-type mice. We found that in vitro insulin-stimulated Akt phosphorylation was normal in soleus muscle from mice with one functional allele of ATM (ATM +/–) and from ATM –/– mice. However, insulin did not stimulate glucose transport or phosphorylation of AS160 in ATM –/– soleus. ATM protein level was markedly higher in wild type EDL than in wild type soleus. In extensor digitorum longus (EDL) from ATM –/– mice, insulin did not stimulate glucose transport. However, in contrast to findings for soleus, insulin-stimulated Akt phosphorylation was blunted in ATM –/– EDL, concomitant for a tendency for insulin-stimulated phosphatidylinositol 3-kinase activity to be decreased. Together, the findings suggest that ATM plays a role in insulin-stimulated glucose transport at the level of AS160 in muscle comprised of slow and fast oxidative-glycolytic fibers (soleus) and at the level of Akt in muscle containing fast glycolytic fibers (EDL).

Keywords

Akt; GLUT4; AS160; fiber type; insulin; skeletal muscle

Introduction

Ataxia telangiectasia mutated (ATM) has well-known nuclear roles, such as marshaling responses to double-stranded DNA breaks (Bhatti et al. 2011). However, ATM can also be cytosolic and has been found to have a number of acute, non-nuclear functions, such as sensing of oxidative stress, prevention of apoptosis, and regulation of cytochrome *c* oxidase activity (Bhatti et al. 2011; Patel et al. 2011). Of greatest relevance to this study, ATM has been shown to be activated by insulin and to contribute to aspects of insulin action such as phosphorylation of 4EBP1 (Yang and Kastan 2000). In particular, it appears that ATM is required for insulin-stimulated phosphorylation of Akt in 293T cells (a line derived from human embryonic kidney), Cos cells (a fibroblast-like line derived from monkey kidney),

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human fibroblasts, mouse embryonic fibroblast cells, and C2C12 myotubes (Halaby et al. 2008; Jeong et al. 2010; Viniegra et al. 2005). Notably, activation of Akt is central to insulin-stimulated glucose transport in skeletal muscle (Foley et al. 2011), which is the primary depot for insulin stimulated clearance of blood glucose (Shulman et al. 1990). Intriguingly, insulin-stimulated glucose transport is suppressed in L6 myotubes and C2C12 myotubes by inhibition of ATM (Jeong et al. 2010) and in L6 myotubes expressing kinase dead ATM (Halaby et al. 2008). On the other hand, Hresko and Mueckler reported that in the insulin-responsive 3T3-L1 adipocyte cell line, knockdown of ATM protein did not interfere with insulin-stimulated Akt phosphorylation (Hresko and Mueckler 2005). Similarly, in an experiment performed with a small sample size, inhibition of ATM did not interfere with insulin-stimulated Akt phosphorylation in mouse soleus (Jeong et al. 2010).

While data from cultured muscle cells suggest a role of ATM in regulation of insulin action (Halaby et al. 2008; Jeong et al. 2010), to our knowledge, there has been no previous study of whether ATM plays an acute role in insulin-stimulated glucose uptake in skeletal muscle. Given the predominance of skeletal muscle in insulin-stimulated blood glucose disposal (DeFronzo 1988; Shulman et al. 1990), it is important to determine whether there is a role of ATM in regulation of insulin stimulated glucose transport in skeletal muscle. Intriguingly, long-term treatment of mice with chloroquine, a compound that activates ATM, improves glucose tolerance (Schneider et al. 2006), suggesting a role of ATM in insulin action. Although there are discrepant reports regarding whether or not ATM plays a role in insulin-stimulated activation of Akt, we gave the benefit of the doubt to the original report by Viniegra et al using Cos and 293T cells (Viniegra et al. 2005) and a separate study using L6 myoblasts (Halaby et al. 2008), both of which demonstrated a role of ATM in insulin action. Thus, we hypothesized that insulin-stimulated Akt phosphorylation and glucose transport would be impaired in skeletal muscle of animals that are genetically deficient in ATM.

Materials and Methods

Materials

Antibodies against Akt, Akt phosphorylated on S473 (P-Akt S473), P-Akt T308, AS160, phosphorylated substrates of Akt (P-AS), IRS-1, P-IRS-1 S302, and P-IRS-1 S612 were obtained from Cell Signaling Technology (Danvers, MA). Antibodies against ATM were obtained from Sigma-Aldrich (St. Louis, MO). Antibodies against GLUT4 were a generous gift from Michael Mueckler (Washington University). Horseradish peroxidase-conjugated goat anti-rabbit antibodies were obtained from ThermoFisher (Rockford, IL). Radiolabeled mannitol and 2-deoxyglucose were from American Radiolabeled Chemicals, Inc. (St. Louis, MO). Insulin (Humulin) was produced by Eli Lilly and Company (Indianapolis, IN).

Animals

All work with live animals was done with approval of the Institutional Animal Care and Use Committee of Saint Louis University. Mice that were heterozygous for a truncation mutation of ATM (Barlow et al. 1996) were purchased from Jackson Laboratory (Bar Harbor, ME). Animals were housed in a facility with a 12 hour light-dark cycle and given free access to food and water. Mice that were heterozygous for the mutation (ATM +/-) were bred to obtain wild-type (ATM +/+) and homozygous mutant (ATM -/-) animals (7.9 ± 0.6 weeks of age at the time of procedures). As described by Miles et al (Miles et al. 2007), it appears that peripheral insulin resistance occurs in young ATM -/- mice (3 months old). This is compounded by impaired insulin secretion in aged ATM -/- mice. Thus, we chose to use younger mice to avoid the possible confounding effects of long-term hyperglycemia and hypoinsulinemia that become apparent in the older ATM-deficient animals.

Genotyping of each individual animal was performed with DNA isolated from tail samples using primers described by Barlow et. al. (Barlow et al. 1996). Briefly, PCR was run with two primer sets: one that would produce a PCR product from a wild-type allele of ATM, and another that would produce a larger PCR product only for an ATM allele containing the truncation mutation. The data in this paper were obtained from 92 animals, both male and female. No gender differences in outcome measures were noted.

Muscle incubations

In vitro muscle incubations were performed as previously described (Fisher et al. 2002; Ju et al. 2004; Smith J.L. et al. 2004). Mice were anesthetized intraperitoneally with sodium pentobarbital (50 mg/kg), and soleus or extensor digitorum longus (EDL) muscles were excised and incubated for one hour at 35 °C in Krebs-Henseleit Buffer (KHB) containing 8 mM glucose, 32 mM mannitol, and 0.1% radioimmunoassay-grade bovine serum albumin (BSA) in vials gassed with 95% O₂:5% CO₂. Muscles were then incubated for 30 min in the same buffer in the absence or presence of 2 mU/ml (~12 nM, a supramaximal insulin concentration) insulin or 60 µU/ml insulin (a submaximal concentration in the physiological range) before they were clamp-frozen with tongs cooled in liquid nitrogen and stored at -80 °C. Other muscles were washed twice at 30 °C for 10 min in glucose-free medium in the absence or presence of insulin and then incubated at 30 °C for 10 min in glucose transport assay medium in the absence or presence of insulin before they were clamp-frozen. Glucose transport assay medium contained 4 mM 2-deoxyglucose (2DG), 2 µCi/ml 3H-2DG, 36 mM mannitol, 0.3 µCi/ml 14C-mannitol, 2 mU/ml insulin if it had been present in previous steps, and 0.1% BSA in KHB.

Western blot analyses

Muscles were homogenized in Kontes ground glass tubes in ice cold buffer (50 mM HEPES with pH 7.4, 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1.5 mM MgCl₂, 1 mM EDTA, 10 mM Na₃PO₄, 100 mM NaF, 2 mM Na₃VO₄, 10 µg/mL leupeptin, 0.5 µg/mL pepstatin, 10 µg/mL aprotinin, and 1 mM phenylmethylsulfonyl fluoride). Homogenates were centrifuged at 4 °C for 10 min at 14,000 rpm. Pellets were discarded, and supernatant protein concentration was assayed by the bicinchoninic acid method (Thermo Fisher, Rockford, IL). For analysis of proteins except for GLUT4 and ATM, supernatants were diluted in Laemmli Sample Buffer containing dithiothreitol, heated for 5 min at 95 °C, and run on 4–20% Tris-HEPES polyacrylamide gels (Thermo Fisher, Rockford, IL). Preparation of samples for GLUT4 blots has been previously described (Ju et al. 2004). For ATM blots, samples were run on 3–8% gels (Invitrogen) with HiMark (Invitrogen) high molecular weight markers. Proteins were transferred to nitrocellulose, blocked in Tris-buffered saline containing 0.1% Tween (TBST) and 5% nonfat dry milk, incubated with primary antibodies, washed several times with TBST, incubated with secondary antibodies, and washed several times before detection with enhanced chemiluminescence (West Lightning, PerkinElmer, Boston, MA). The P-AS band that ran to the same spot as the AS160 band was selected for quantification. In our hands, the TBC1D1 band that cross-reacts with P-AS runs to a distinctly lower level than AS160, so it does not interfere with our selection of the proper P-AS band for AS160. Band volume densities were quantified using TotalLab software (Nonlinear Dynamics, Newcastle upon Tyne, UK). Phosphorylated proteins were normalized to total proteins. For example, P-Akt was normalized to Akt, P-AS was normalized to AS160, and P-IRS-1 was normalized to IRS-1. ATM was normalized to GAPDH, and GLUT4 was normalized to GAPDH or tubulin. Data for western blots were expressed as relative (arbitrary) units (AU) compared to a control group for each data set.

Analysis of 2-deoxyglucose uptake

Muscles were homogenized and protein content was determined as described above. Aliquots were added to Ultima Gold XR scintillation fluid (Perkin Elmer, Boston, MA) and assessed by scintillation counting (TriCarb 3110TR, Perkin Elmer) of muscle samples and samples of the incubation media. Extracellular volume was calculated using disintegrations per minute (DPM) of the ^{14}C -mannitol, which is not membrane permeant. Intracellular 2DG levels were then determined after accounting for 3H DPM in the extracellular space, and 2DG uptake rates were expressed as nmol 2DG/mg protein/10 minutes.

Phosphatidylinositol 3-kinase (PI3K) activity

PI3K activity was assayed using a kinase reaction kit (Echelon Biosciences Inc., Salt Lake City, UT) with competitive ELISA detection of PI3K product, following the manufacturer's instructions.

Statistical methods

Mean comparisons were performed by ANOVA ($\alpha=0.05$) followed by LSD post-hoc analyses when warranted by the ANOVA. Data are presented as means with standard errors.

Results

Insulin-stimulated Akt phosphorylation in soleus muscle

It has been reported that ATM protein levels are substantially decreased in gastrocnemius rats fed a high fat diet, and this decline in ATM occurs concomitant with impaired insulin-stimulated Akt phosphorylation (Halaby et al. 2008). To determine if a physiologically relevant reduction of ATM protein might play a role in decreasing stimulation of Akt phosphorylation by insulin, we compared insulin action in soleus muscles from wild-type mice (ATM $+/+$) and mice that were heterozygous (ATM $+/-$) for a truncation mutation of ATM that produces a protein lacking the kinase domain and thus prevents ATM activity (Barlow et al. 1996). We probed soleus samples with an antibody against ATM that detects full-length ATM but not the truncated ATM protein expressed in animals with one or two mutated ATM alleles. Each individual mouse was genotyped as shown by the examples in figure 1A. As shown in figure 1B, soleus from ATM $+/-$ mice has about half the abundance of ATM protein as soleus from ATM $+/+$ mice ($p<0.05$). ATM protein is not detectable in ATM $-/-$ soleus (figure 1B). As shown in figures 1C and 1D, insulin caused an increase in Akt phosphorylation at S473 and T308 ($p<0.05$) that was similar in soleus from wild-type mice and soleus from mice that were heterozygous for a truncation mutation of ATM.

To further investigate a potential role of ATM in regulation of insulin-stimulated Akt phosphorylation in skeletal muscle, we turned to mice that were completely deficient in functional ATM. As shown in figures 1E and 1F, soleus incubated in the presence of insulin had increased P-Akt S473 and P-Akt T08 regardless of ATM genotype ($p<0.05$), and insulin-stimulated phosphorylation of Akt was not different between ATM $+/+$ and ATM $-/-$ groups.

Total Akt levels (normalized to mg protein) in soleus were not different between ATM $+/+$ and ATM $+/-$ mice (1.00 ± 0.07 vs. 1.03 ± 0.11 , relative units). Likewise, total Akt levels were similar in soleus from ATM $+/+$ and ATM $-/-$ mice (1.00 ± 0.15 vs. 0.99 ± 0.12 , relative units).

In summary, data from figure 1 demonstrate that insulin-stimulated Akt phosphorylation is normal in soleus muscle from mice that were haploinsufficient for ATM and in soleus muscle from mice completely lacking functional ATM.

Insulin-stimulated glucose uptake in soleus muscle

We had previously found that insulin-stimulated Akt phosphorylation was normal in L6 myotubes in the presence of an ATM inhibitor (Jeong et al. 2010) at a concentration that was low but sufficient to inhibit ATM in L6 myotubes (Ching et al. 2010). However, despite no effect on Akt phosphorylation, inhibition of ATM decreased insulin-stimulated glucose transport in L6 myotubes (Jeong et al. 2010).

Thus, although insulin-stimulated Akt phosphorylation was normal in soleus from ATM-deficient mice in the current study (figure 1C–F), it was imperative to determine if there was an influence of ATM downstream of Akt. Accordingly, we assessed insulin-stimulated glucose transport in soleus muscles from ATM $-/-$ animals. As shown in figure 2A, insulin increased glucose transport in soleus muscles from wild type animals ($p < 0.005$), but insulin action was attenuated in soleus from ATM $-/-$ mice. In the presence of insulin, glucose transport was about two-fold higher for wild type mice than for ATM $-/-$ mice ($p < 0.005$). However, GLUT4 protein levels were not different between wild-type and ATM $-/-$ mice (figure 2B).

Phosphorylation of AS160 in soleus muscle

Because glucose transport was decreased in muscle from ATM $-/-$ animals despite normal phosphorylation of Akt and normal levels of GLUT4, we next looked downstream of Akt to the rab GTPase activating protein (GAP) AS160. AS160 has been implicated in preventing sorting of GLUT4 vesicles to exocytotic pathways, and phosphorylation of AS160 prevents its interference with GLUT4 exocytosis (Foley et al. 2011). We hypothesized that AS160 phosphorylation in the presence of insulin would be decreased in soleus muscles lacking functional ATM. As shown in figure 2C, insulin increased phosphorylation of AS160 in soleus from wild-type animals ($p < 0.05$) but not soleus from ATM $-/-$ animals.

The ANOVA for insulin effects on AS160 in soleus wild-type and ATM $+/-$ muscle was nearly significant ($p = 0.07$, figure 2D), so we performed post hoc comparisons. With the caveat that the ANOVA was not quite significant, insulin caused an increase of AS160 phosphorylation in wild-type soleus ($p < 0.05$) but not in ATM $+/-$ soleus, and insulin-stimulated AS160 phosphorylation was higher in soleus from ATM $+/+$ mice than soleus from ATM $+/-$ mice ($p < 0.05$).

Insulin action in fast twitch muscle

Skeletal muscle of rodents contains three general fiber types: slow, fast oxidative-glycolytic, and fast glycolytic. Soleus muscle of mice is about 60% slow and 40% fast oxidative-glycolytic (Burkholder et al. 1994). Thus, the findings for soleus shown in figures 1 and 2 suggest that ATM does not play a role in insulin-stimulated phosphorylation of Akt for either slow or fast oxidative-glycolytic skeletal muscle, the fiber types present in soleus (Burkholder et al. 1994). To determine whether ATM plays a role in insulin-stimulated phosphorylation of Akt in fast glycolytic muscle, we repeated procedures using extensor digitorum longus (EDL) muscle, which has about 50% fast oxidative-glycolytic and 50% fast glycolytic fibers (Burkholder et al. 1994).

ATM is not detectable in ATM $-/-$ EDL (figure 3A). As shown in figure 3B, insulin increased glucose transport in wild-type EDL muscle ($p < 0.05$) but not in ATM-deficient EDL, despite normal GLUT4 content in ATM-deficient EDL (figure 3C). However, in contrast to results for soleus, insulin-stimulated Akt phosphorylation was blunted in ATM-deficient EDL to about half the level in wild-type EDL ($p < 0.05$ for comparison of means for insulin-stimulated $+/+$ vs. $-/-$ EDL, figure 4D). Thus, it appears that in EDL from ATM-deficient mice, blunted insulin-stimulated Akt phosphorylation might contribute to

prevention of insulin-stimulated glucose transport. We next looked upstream of Akt and found that insulin-stimulated PI3K phosphorylation tended (not statistically significant, $p=0.196$) to be decreased in ATM $-/-$ EDL compared to wild-type EDL (figure 3E). As it has been suggested that increased serine phosphorylation of IRS-1 in ATM-deficient tissue impairs insulin signaling, we looked at IRS-1 serine phosphorylation at S302 and S612. S302 phosphorylation was not different between genotypes. There were no significant differences among groups for S612 phosphorylation (figure 3F, ANOVA p -value = 0.320). However, when muscles incubated in the absence of insulin and the presence of insulin were pooled, comparing S612 by genotype, S612 phosphorylation was greater for ATM $-/-$ EDL than wild-type EDL ($p<0.05$).

Finally, given the muscle-type-dependent influence of ATM on insulin-stimulated Akt phosphorylation, we compared ATM protein levels in soleus and EDL. ATM protein level (normalized to GAPDH) was about 10-fold higher in EDL than in soleus ($p<0.05$, figure 3G).

We next determined insulin action under conditions that were as physiologically relevant as possible. As shown in figure 1B, ATM protein levels are about 50% lower in ATM $+/-$ soleus than in wild-type soleus. This lower ATM level is similar to the decrease in muscle ATM protein level in muscle of rats fed a high fat diet (Halaby et al). Thus, the amount of ATM protein in ATM $+/-$ soleus represents a decreased level of ATM that is in the physiological range. Using wild-type and ATM $+/-$ animals, we determined insulin signaling parameters in muscle exposed to a submaximal concentration of insulin in the physiological range (60 μ U/ml). As shown in figure 4A, insulin-stimulated Akt phosphorylation was normal in ATM $+/-$ soleus. ATM protein levels were not decreased by ATM haploinsufficiency, suggesting that one wild-type ATM allele is sufficient to maintain ATM protein levels in EDL. Given that ATM levels were not affected by ATM haploinsufficiency in EDL, it is not surprising that Akt phosphorylation was also normal in ATM $+/-$ EDL (figure 4C),

Discussion

The new information provided by this study is that ATM plays a role in regulation of insulin stimulated glucose transport in skeletal muscle, the most important storage depot for glucose cleared from the blood in the presence of insulin (DeFronzo 1988; Shulman et al. 1990). This lends credence to the suggestion (Yang et al. 2011) that chemical activation of ATM could be a means for increasing insulin action. In EDL muscle, the role of ATM appears to be upstream of Akt, leading to impaired phosphorylation of Akt in ATM-deficient EDL in response to a maximal insulin concentration. In contrast, insulin-stimulated AS160 phosphorylation appears decreased in ATM $-/-$ soleus despite normal Akt phosphorylation.

It appears that the mechanism for ATM's influence on insulin-stimulated glucose transport is not as straightforward as might be surmised from previous findings regarding the role of ATM in insulin-stimulated activation of Akt in some cell types (Halaby et al. 2008; Jeong et al. 2010; Viniegra et al. 2005). One of the most striking findings of the current study is the fiber type dependence for a role of ATM in insulin-stimulated Akt phosphorylation. From a comparison of results in soleus and EDL, it appears that ATM plays a role in insulin-stimulated Akt phosphorylation only in fast glycolytic fibers, which are present in EDL but not in soleus. In contrast, insulin-stimulated Akt phosphorylation is normal in ATM-deficient soleus muscle, which contains slow and fast oxidative-glycolytic fibers.

While a mechanism for the fiber type dependence of ATM's role in insulin action is beyond the scope of this study, it appears that ATM has an expanded metabolic role in fast

glycolytic muscle—regulation of both Akt and glucose transport—compared to the fiber types in soleus. Intriguingly, patients with type 2 diabetes have greater percentages of fast glycolytic fibers than age- and BMI-matched controls (Oberbach et al. 2006), suggesting the possibility of a key role for ATM in muscle in these subjects. The observation of Halaby et al (Halaby et al. 2008) of the association between decreased ATM protein and decreased insulin-stimulated Akt phosphorylation in gastrocnemius from fat-fed rats is consistent with the current findings of blunted insulin action in ATM-deficient EDL, as fast glycolytic fibers make up the bulk of rat gastrocnemius (Delp and Duan 1996).

Stimulation of glucose transport in muscle involves activation of Akt and phosphorylation of Rab GTPase activating protein AS160 by Akt (Foley et al. 2011). Phosphorylation of AS160 prevents it from suppressing Rab activity and allows plasma membrane localization of GLUT4 (Foley et al. 2011). The current data from soleus and previous data obtained with chemical inhibition of ATM in soleus (Jeong et al. 2010) suggest that ATM plays a role in altering AS160 phosphorylation in response to insulin. It seems unlikely that ATM directly phosphorylates AS160 on one of the Akt target sites of AS160, as none of these sites (Sano et al. 2003) contains the signature serine-glutamine (SQ) or threonine-glutamine (TQ) motifs that are characteristic of ATM targets (O'Neill et al. 2000). On the other hand, the SQ motif is present in three known phosphorylation sites of AS160: S66, S285, and S1126 (Wang et al. 2007) (Hsu et al. 2011; Trebak et al. 2010). Notably, S66 lies squarely in the middle of a PH domain of AS160, as detected with the Conserved Domain Database (Marchler-Bauer et al. 2011). One could speculate that by phosphorylation of this site or another SQ site, the localization of AS160 might be altered, so that it would be less accessible to Akt.

Since the work of Viniegra et al (Viniegra et al. 2005) and Hresko and Mueckler (Hresko and Mueckler 2005), it has been apparent that there are cell type differences for a role of ATM in regulation of insulin stimulated Akt phosphorylation. For example, insulin caused an increase in Akt phosphorylation in Cos cells expressing wild type ATM but not in cells expressing kinase dead ATM (Viniegra et al. 2005). Furthermore, expression of the constitutively active kinase domain of ATM was sufficient to increase Akt phosphorylation (Viniegra et al. 2005). Conversely, the normal increase in Akt Serine 473 phosphorylation in response to insulin in human fibroblasts did not occur in ATM-deficient cells (Viniegra et al. 2005). Thus, the findings described above suggest that ATM plays a role in insulin-stimulated phosphorylation of Akt. In contrast, in 3T3-L1 adipocytes, an 85% decrease in ATM protein mediated by siRNA against ATM had no effect on insulin-stimulated Akt phosphorylation (Hresko and Mueckler 2005). It is notable that Hresko and Mueckler selected 3T3-L1 adipocytes as their experimental platform because of the responsiveness of the cell line to insulin in terms of glucose transport (Hresko and Mueckler 2005). This idea appears to be instructive, as ATM plays a role insulin-stimulated Akt phosphorylation for cells not considered to be greatly insulin-responsive, such as Cos cells, 293T cells, human fibroblasts, mouse embryonic fibroblasts, human rhabdomyosarcoma cells, L6 myoblasts, and C2C12 myotubes (Halaby et al. 2008; Jeong et al. 2010; Viniegra et al. 2005). In a more insulin responsive cell culture model, L6 myotubes, inhibition of ATM did not affect insulin-stimulated Akt phosphorylation, though it still prevented insulin-stimulated glucose transport (Jeong et al. 2010). Based on the current findings for skeletal muscle, there appears to be a role for ATM in insulin-stimulated Akt phosphorylation in the less insulin-responsive EDL but not in the more insulin-responsive soleus.

The higher levels of ATM protein present in EDL than in soleus could be a possible explanation for a meaningful role of ATM in insulin action in EDL but not soleus. On the other hand, it has been shown that IGF-1-stimulated Akt phosphorylation is impaired in ATM +/- soleus (Ching et al. 2012b). Thus, at least in terms of IGF-1 action, the amount of ATM protein in soleus is sufficient to make an impact on Akt. It seems possible, therefore,

that a role of ATM in activation of Akt is dependent on both ATM protein abundance and the specific receptor (insulin receptor or IGF-1 receptor) that has activated the signaling pathway.

The current data add to the body of literature regarding ATM and insulin action. It has been shown that ATM $-/-$ mice become hyperglycemic compared to wild-type mice by 21–27 weeks of age, concomitant with decreased insulin secretion in ATM $-/-$ mice (Miles et al. 2007). However, in 3 month old animals, plasma glucose levels are higher in ATM $-/-$ mice than in wild type animals during oral glucose tolerance tests (Miles et al. 2007). The hyperglycemia occurred despite equal or higher insulin levels in the ATM-deficient animals compared to the wild type mice, suggesting that insulin-stimulated blood glucose disposal was blunted in these animals. These animals were the same line that was used in the current study, so the findings are particularly relevant. The OGTT findings (Miles et al. 2007) and the current data together suggest that impaired glucose tolerance in ATM $-/-$ mice could be in part a result of attenuated insulin action in skeletal muscle.

In contrast to findings of decreased insulin action in ATM-deficient mice, in ApoE $-/-$ mice, chronic treatment with the ATM activator chloroquine improves oral glucose tolerance, enhances the hypoglycemic response to insulin injection, and markedly improves insulin-stimulated Akt phosphorylation in liver (Razani et al. 2010). Similarly, chronic chloroquine treatment lowered fasting and fed plasma glucose levels in ob/ob mice (Schneider et al. 2006). Furthermore, for mice on an ApoE $-/-$ background, chloroquine lowered the glucose area under the curve for an intraperitoneal glucose tolerance test in ATM $+/+$ animals but not ATM $-/-$ mice (Schneider et al. 2006). The current data suggest that some of these effects of ATM on glucose metabolism could be related to a role of ATM in regulation of glucose transport in skeletal muscle.

Schneider et al (Schneider et al. 2006) studied insulin signaling in ATM-deficient mice on an ApoE $-/-$ background. They found that liver IRS-2-associated PI3K activity was decreased by about a third in ATM $+/+$ mice compared to ATM $+/+$ mice. In aorta, phosphorylation of IRS-1 at S302 (the corresponding site in human IRS-1 is S307) was increased in ATM $-/-$ mice. Taking these data together, Schneider et al proposed that decreased ATM activity leads to increased serine phosphorylation of IRS-1 with a subsequent decrease in insulin signaling downstream of IRS-1. Consistent with this, IGF-1-stimulated PI3K activity is decreased in ATM $+/+$ muscle even though IGF-1-stimulated tyrosine phosphorylation of IRS-1 is normal in ATM $+/+$ muscle (Ching et al. 2012). The current findings suggest that insulin signaling is impaired upstream of Akt in ATM-deficient EDL,

In summary, ATM plays a role in insulin-stimulated glucose transport in skeletal muscle. In muscle containing slow and fast oxidative-glycolytic fibers, ATM appears to influence AS160 phosphorylation without affecting Akt. However, ATM appears necessary to full insulin-stimulated phosphorylation of Akt in muscle that contains fast glycolytic fibers. These findings for a role of ATM in regulation of insulin action in skeletal muscle could help explain the insulin resistance found in humans who lack functional ATM (Blevins, Jr. and Gebhart 1996; Schalch et al. 1970) and provides support for future study regarding the suggested use of ATM-activating drugs to treat diabetes (Yang et al. 2011).

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References

- Barlow C, Hirotsune S, Paylor R, Liyanage M, Eckhaus M, Collins F, et al. Atm-deficient mice: a paradigm of ataxia telangiectasia. *Cell*. 1996; 86:159–171.10.1016/S0092-8674(00)80086-0 [PubMed: 8689683]
- Bhatti S, Kozlov S, Farooqi AA, Naqi A, Lavin M, Khanna KK. ATM protein kinase: the linchpin of cellular defenses to stress. *Cell Mol Life Sci*. 2011; 68:2977–3006.10.1007/s00018-011-0683-9 [PubMed: 21533982]
- Blevins LS Jr, Gebhart SS. Insulin-resistant diabetes mellitus in a black woman with ataxia-telangiectasia. *South Med J*. 1996; 89:619–621. [PubMed: 8638204]
- Burkholder TJ, Fingado B, Baron S, Lieber RL. Relationship between muscle fiber types and sizes and muscle architectural properties in the mouse hindlimb. *J Morphol*. 1994; 221:177–190.10.1002/jmor.1052210207 [PubMed: 7932768]
- Ching JK, Luebbert SH, Collins RL IV, Zhang Z, Marupudi N, Banerjee S, et al. ATM impacts IGF-1 signaling in skeletal muscle. *Exp Physiol*. 2012 (in press). 10.1113/expphysiol.2012.066357
- Ching JK, Rajguru P, Marupudi N, Banerjee S, Fisher JS. A role for AMPK in increased insulin action after serum starvation. *Am J Physiol Cell Physiol*. 2010; 299:C1171–C1179.10.1152/ajpcell.00514.2009 [PubMed: 20810907]
- Defronzo RA. Lilly lecture 1987. The triumvirate: beta-cell, muscle, liver A collusion responsible for NIDDM. *Diabetes*. 1988; 37:667–687. [PubMed: 3289989]
- Delp MD, Duan C. Composition and size of type I, IIA, IID/X and IIB fibers and citrate synthase activity of rat muscle. *J Appl Physiol*. 1996; 80:261–270. [PubMed: 8847313]
- Fisher JS, Nolte LA, Kawanaka K, Han DH, Jones TE, Holloszy JO. Glucose transport rate and glycogen synthase activity both limit skeletal muscle glycogen accumulation. *Am J Physiol Endocrinol Metab*. 2002; 282:1214–1221.10.1152/ajpendo.00254.2001
- Foley K, Boguslavsky S, Klip A. Endocytosis, recycling, and regulated exocytosis of glucose transporter 4. *Biochemistry*. 2011; 50:3048–3061.10.1021/bi2000356 [PubMed: 21405107]
- Halaby MJ, Hibma JC, He J, Yang DQ. ATM protein kinase mediates full activation of Akt and regulates glucose transporter 4 translocation by insulin in muscle cells. *Cell Signal*. 2008; 20:1555–1563.10.1016/j.cellsig.2008.04.011 [PubMed: 18534819]
- Hresko RC, Mueckler M. mTOR/RICTOR is the Ser473 kinase for Akt/PKB in 3T3-L1 adipocytes. *J Biol Chem*. 2005; 280:40406–40416.10.1074/jbc.M508361200 [PubMed: 16221682]
- Hsu PP, Kang SA, Rameseder J, Zhang Y, Ottina KA, Lim D, et al. The mTOR-regulated phosphoproteome reveals a mechanism of mTORC1-mediated inhibition of growth factor signaling. *Science*. 2011; 332:1317–1322.10.1126/science.1199498 [PubMed: 21659604]
- Jeong I, Patel AY, Zhang Z, Patil PB, Nadella ST, Nair S, et al. Role of ataxia telangiectasia mutated in insulin signaling of muscle-derived cell lines and mouse soleus. *Acta Physiologica*. 2010; 198:465–475.10.1111/j.1748-1716.2009.02069.x [PubMed: 20003097]
- Ju JS, Smith JL, Oppelt PJ, Fisher JS. Creatine feeding increases GLUT4 expression in rat skeletal muscle. *Am J Physiol Endocrinol Metab*. 2004; 288:E347–E352.10.1152/ajpendo.00238.2004 [PubMed: 15494613]
- Marchler-Bauer A, Lu S, Anderson JB, Chitsaz F, Derbyshire MK, DeWeese-Scott C, et al. CDD: a Conserved Domain Database for the functional annotation of proteins. *Nucleic Acids Res*. 2011; 39:D225–D229.10.1093/nar/gkq1189 [PubMed: 21109532]
- Miles P, Treuner K, Latronica M, Olefsky JM, Barlow C. Impaired insulin secretion in a mouse model of Ataxia Telangiectasia. *Am J Physiol Endocrinol Metab*. 2007; 293:E70–74.10.1152/ajpendo.00259.2006 [PubMed: 17356010]
- O'Neill T, Dwyer AJ, Ziv Y, Chan DW, Lees-Miller SP, Abraham RH, et al. Utilization of oriented peptide libraries to identify substrate motifs selected by ATM. *J Biol Chem*. 2000; 275:22719–22727.10.1074/jbc.M001002200 [PubMed: 10801797]

- Oberbach A, Bossenz Y, Lehmann S, Niebauer J, Adams V, Paschke R, et al. Altered fiber distribution and fiber-specific glycolytic and oxidative enzyme activity in skeletal muscle of patients with type 2 diabetes. *Diabetes Care*. 2006; 29:895–900.10.2337/diacare.29.04.06.dc05-1854 [PubMed: 16567834]
- Patel AY, McDonald TM, Spears LD, Ching JK, Fisher JS. Ataxia telangiectasia mutated influences cytochrome c oxidase activity. *Biochem Biophys Res Commun*. 2011; 405:599–603.10.1016/j.bbrc.2011.01.075 [PubMed: 21266166]
- Razani B, Feng C, Semenkovich CF. p53 is required for chloroquine-induced atheroprotection but not insulin sensitization. *J Lipid Res*. 2010; 51:1738–1746.10.1194/jlr.M003681 [PubMed: 20208057]
- Sano H, Kane S, Sano E, Miinea CP, Asara JM, Lane WS, et al. Insulin-stimulated phosphorylation of a Rab GTPase-activating protein regulates GLUT4 translocation. *J Biol Chem*. 2003; 278:14599–14602.10.1074/jbc.C300063200 [PubMed: 12637568]
- Schalch DS, McFarlin DE, Barlow MH. An unusual form of diabetes mellitus in ataxia telangiectasia. *N Engl J Med*. 1970; 282:1396–1402.10.1056/NEJM197006182822503 [PubMed: 4192270]
- Schneider JG, Finck BN, Ren J, Standley KN, Takagi M, Maclean KH, et al. ATM-dependent suppression of stress signaling reduces vascular disease in metabolic syndrome. *Cell Metab*. 2006; 4:377–389.10.1016/j.cmet.2006.10.002 [PubMed: 17084711]
- Shulman GI, Rothman DL, Jue T, Stein P, DeFronzo RA, Shulman RG. Quantitation of muscle glycogen synthesis in normal subjects and subjects with non-insulin-dependent diabetes by ¹³C nuclear magnetic resonance spectroscopy. *N Engl J Med*. 1990; 322:223–228.10.1056/NEJM199001253220403 [PubMed: 2403659]
- Smith JL, Ju JS, Saha BM, Racette BA, Fisher JS. Levodopa with carbidopa diminishes glycogen concentration, glycogen synthase activity, and insulin-stimulated glucose transport in rat skeletal muscle. *J Appl Physiol*. 2004; 97:2339–2346.10.1152/jappphysiol.01219.2003 [PubMed: 15258132]
- Trebbak JT, Taylor EB, Witczak CA, An D, Toyoda T, Koh HJ, et al. Identification of a novel phosphorylation site on TBC1D4 regulated by AMP-activated protein kinase in skeletal muscle. *Am J Physiol Cell Physiol*. 2010; 298:C377–C385.10.1152/ajpcell.00297.2009 [PubMed: 19923418]
- Viniegra JG, Martinez N, Modirassari P, Losa JH, Parada CC, Lobo VJ, et al. Full activation of PKB/Akt in response to insulin or ionizing radiation is mediated through ATM. *J Biol Chem*. 2005; 280:4029–4036.10.1074/jbc.M410344200 [PubMed: 15546863]
- Wang Y, Ding SJ, Wang W, Jacobs JM, Qian WJ, Moore RJ, et al. Profiling signaling polarity in chemotactic cells. *Proc Natl Acad Sci U S A*. 2007; 104:8328–8333.10.1073/pnas.0701103104 [PubMed: 17494752]
- Yang DQ, Halaby MJ, Li Y, Hibma JC, Burn P. Cytoplasmic ATM protein kinase: an emerging therapeutic target for diabetes, cancer and neuronal degeneration. *Drug Discov Today*. 2011; 16:332–338.10.1016/j.drudis.2011.02.001 [PubMed: 21315178]
- Yang DQ, Kastan MB. Participation of ATM in insulin signalling through phosphorylation of eIF-4E-binding protein 1. *Nat Cell Biol*. 2000; 2:893–898.10.1038/35046542 [PubMed: 11146653]

Figure 1. Insulin-stimulated phosphorylation of Akt in soleus muscle

A) Soleus muscles from mice with one non-functional allele of ATM (ATM +/-), two non-functional alleles of ATM (ATM -/-), and wild-type littermates (ATM +/+) were genotyped from tail DNA by PCR using primers specific to wild-type ATM and truncated ATM. Presence of only the larger PCR product was characteristic of ATM -/- mice, presence of only the smaller PCR product indicated a wild-type animal, and presence of both bands identified ATM +/- animals. B) Western blot of ATM normalized to GAPDH ($\dagger p < 0.05$, $n = 10-11$ /group). C-F) Soleus muscles were incubated for 30 min in vitro in the absence or presence of 2 mU/ml insulin before western blot analysis. C,D) P-Akt S473, P-Akt T308, and Akt in wild-type and ATM +/- mice ($n = 5$ /group). E,F) P-Akt S473, P-Akt T308, and Akt in wild-type and ATM -/- mice ($n = 6$ /group). Data represent means and standard errors. *statistically significant effect of insulin, $p < 0.05$.

Figure 2. Impaired insulin-stimulated glucose transport and AS160 phosphorylation in ATM-deficient soleus muscle

Soleus muscles from wild-type mice and mice that were homozygous for a truncation mutation of ATM (ATM $-/-$) were incubated in vitro in the absence or presence of 2 mU/ml insulin and then A) assayed for 2-deoxyglucose (2DG) transport ($n=5$ /group) or B) GLUT4 content of soleus from wild-type and ATM-deficient mice. In C, blots were probed with antibodies that bind phosphorylated substrates of Akt (P-AS), and the P-AS band corresponding to the position of AS160 was quantified ($n=8-9$ /group) * or † indicates both a significant insulin effect in ATM $+/+$ muscle and a greater value in insulin-stimulated ATM $+/+$ muscle than in insulin-stimulated ATM $-/-$ muscle (* $p<0.005$, † $p<0.05$). D. P-AS/AS160 for wild-type and ATM $+/-$ soleus incubated in the absence or presence of insulin. ‡§The ANOVA for these comparisons was nearly significant ($p=0.07$) and the post hoc comparisons should be viewed in this light. In these comparisons, ‡insulin stimulated increased P-AS/AS160 in wild-type muscle ($p<0.05$), and §P-AS/AS160 was significantly lower in insulin-stimulated ATM $+/-$ soleus than in ATM $+/+$ soleus ($p<0.05$), $n=5$ /group.

Figure 3. Impaired insulin-stimulated glucose transport and Akt phosphorylation in ATM-deficient EDL muscle

EDL muscles from wild-type (ATM ^{+/+}) and ATM-deficient (ATM ^{-/-}) mice were A) probed for ATM and GAPDH. B–G) incubated in vitro in the absence or presence of 2 mU/ml insulin and then assayed for B) 2-deoxyglucose (2DG) transport (n=4–5/group), C) GLUT4 content (n=4/group), D) Akt phosphorylation (C, n=3/group), E) insulin-stimulated PI3K activity (n=3/group), and F) IRS-1 phosphorylation at S302 and S612 (n=3/group). G) Soleus and EDL muscles from wild-type animals were probed for ATM and GAPDH (n=3/group). *indicates both a significant insulin effect in ATM ^{+/+} muscle and a greater value in insulin-stimulated ATM ^{+/+} muscle than in insulin-stimulated ATM ^{-/-} muscle (p<0.05). †greater than basal values for EDL from ATM ^{+/+} animals (p<0.001) and greater than values for insulin-stimulated EDL from ATM ^{-/-} mice (p<0.05). ‡effect of insulin in EDL from ATM ^{-/-} animals (p<0.01). §significant difference (p<0.05).

Figure 4. Insulin signaling in response to a submaximal concentration of insulin

Soleus and EDL muscles from wild-type and ATM +/- mice were incubated for 30 min in 60 μ U/ml insulin and analyzed for A) P-Akt S473 and T308, Akt, P-AS, and AS160 in soleus (n=3/group), B) ATM and GAPDH in EDL (n=10/group), and C) P-Akt S473 and Akt in EDL (n=4–5/group). *significant insulin effect (p<0.05). †tendency toward insulin effect (p=0.07).