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# Chloroquine increases phosphorylation of AMPK and Akt in myotubes

Larry D. Spears<sup>a</sup>, Andrew V. Tran<sup>a</sup>, Charles Y. Qin<sup>a</sup>, Supriya B. Hobbs<sup>a</sup>, Cheryl A. Liang Burns<sup>a</sup>, Nathaniel K. Royer<sup>a</sup>, Zhihong Zhang<sup>b</sup>, Lyle Ralston<sup>b</sup>, Jonathan S. Fisher<sup>a,c,\*</sup>

<sup>a</sup> Department of Biology, Saint Louis University, St. Louis, MO 63103, USA

<sup>b</sup> Sigma-Aldrich, St. Louis, MO 63103, USA

<sup>c</sup> Center for Cardiovascular Research, Saint Louis University, St. Louis, MO 63103, USA

\* Corresponding author. Department of Biology and Center for Cardiovascular Research, Saint Louis University, 3507 Laclede Ave, St. Louis, MO 63103, USA.

E-mail address: [fisherjs@slu.edu](mailto:fisherjs@slu.edu) (J.S. Fisher).

## Abstract

**Aims:** There are reports that ataxia telangiectasia mutated (ATM) can activate the AMP-activated protein kinase (AMPK) and also Akt, two kinases that play integral parts in cardioprotection and metabolic function. We hypothesized that chloroquine and resveratrol, both known ATM activators, would also activate AMPK and Akt.

**Main methods:** Phosphorylation of AMPK and Akt was assessed after C2C12 myotubes were exposed to chloroquine or resveratrol. Additional experiments were done in cells expressing shRNA against ATM or in the presence of the ATM inhibitor KU55933. The effects of chloroquine on intracellular calcium were assessed with the fluorescent probe Calcium Green-1 AM.

**Key findings:** 0.5 mM chloroquine increased AMPK phosphorylation by nearly four-fold ( $P < 0.05$ ), and 0.25 mM chloroquine roughly doubled Akt phosphorylation ( $P < 0.05$ ). Chloroquine also increased autophosphorylation of ATM by ~50% ( $P < 0.05$ ). Resveratrol (0.15 mM) increased AMPK phosphorylation about three-fold ( $P < 0.05$ ) but in contrast to chloroquine sharply decreased Akt phosphorylation. Chloroquine increased AMPK and Akt phosphorylation in myotubes expressing shRNA against ATM that reduced

ATM protein levels by about 90%. Likewise, chloroquine-stimulated phosphorylation of AMPK and Akt and resveratrol-stimulated phosphorylation of AMPK were not altered by inhibition of ATM. Chloroquine decreased intracellular calcium by >50% concomitant with a decrease in glucose transport. **Significance:** These ATM-independent effects of chloroquine on AMPK and Akt and the additional effect to decrease intracellular calcium are likely to partially underlie the positive metabolic effects of chloroquine that have been reported in the literature.

Keywords: Applied sciences, Therapeutics in cell biology, Animal metabolism

## 1. Introduction

Ataxia Telangiectasia (A-T) is an autosomal disease caused by mutations in the ataxia telangiectasia mutated (ATM) gene [1]. The ATM gene codes for a protein that is a serine-threonine kinase and a member of the phosphatidylinositol 3 kinase like kinase (PI3KK) family. In addition to ATM's well-known functions in cell cycle control and response to DNA damage, ATM has increasingly been found to display a variety of metabolic functions. For example, ATM plays antioxidant roles by stimulating the pentose phosphate pathway [2] and through increasing uptake of dehydroascorbic acid (DHA) [3], the oxidized form of the potent antioxidant ascorbate. Mitochondrial dysregulation has been reported for ATM-null thymocytes, with features including disorganized mitochondrial structure, increased mitochondrial reactive oxygen species (ROS), and decreases in whole-cell ATP despite an increase in mitochondrial volume [4]. ATM has also been shown to play a role in insulin-stimulated glucose transport in cultured muscle cells and in skeletal muscle [5,6, 7].

Two critical metabolic regulators in particular, the AMP-activated protein kinase (AMPK) and Akt seem to be central mediators of ATM's metabolic functions. AMPK is a sensor of cellular energy status and responds to metabolic stress by activating diverse responses including an increase in fat oxidation [8] and an increase in expression and activity of mitochondrial proteins [9, 10]. ATM and AMPK appear to work in concert in response to cellular stress. For example, activity of both ATM and AMPK is required for the increase in insulin action in cultured muscle cells in response to serum starvation [11]. Further, activation of AMPK concomitant with ATM has been reported for a wide variety of cell types [12, 13, 14, 15, 16] with stimuli including depletion of mitochondria, hydrogen peroxide, and ionizing radiation. Similarly, activation of Akt—a protein with diverse functions in cell survival, protection of cardiac cells from hypertrophic stimuli, and fuel uptake and storage—by ionizing radiation requires functional ATM [17]. Likewise, activation of Akt by insulin-like growth factor

1 (IGF-1) and insulin is impaired in many cell types by inhibition or genetic ablation of ATM [5, 6, 7, 17, 18].

Chloroquine (CQ), a compound that activates ATM without causing DNA damage [19], has excitingly been shown to protect mice against aortic root atherosclerosis in high-fat fed mice [20]. Long-term CQ treatment also improved glucose tolerance in mouse genetic models of obesity [18] as well as decreased the risk of diabetes in patients with rheumatoid arthritis [21]. To understand the nature of CQ's effects, especially in regard to a role for ATM, it is important to determine whether ATM is required for CQ's actions. Accordingly, we hypothesized that CQ would activate the key metabolic regulators AMPK and Akt and that ATM would be required for these effects. This work also investigated effects of another ATM activator, resveratrol (RE), which like CQ has been reported to improve systemic glucose control in both mice and humans [22, 23].

Surprisingly, in our hands CQ activated both AMPK and Akt even in the presence of an ATM inhibitor or in cells expressing shRNA against ATM. CQ also decreased intracellular calcium levels and did not cause an increase in glucose uptake. Interestingly, while RE increased AMPK phosphorylation, it decreased Akt phosphorylation. Thus, the data suggest that CQ and RE could have metabolic effects beyond simple activation of ATM.

## 2. Materials and methods

### 2.1. Materials

Dulbecco's modified Eagle's medium (DMEM), trypsin/EDTA and phosphate-buffered saline (PBS) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Penicillin/streptomycin (10,000 U penicillin/ml, 10 mg streptomycin/ml, and 0.025 mg fungizone/ml) was purchased from Gemini Bio-Products (Woodland, CA, USA). Antibodies against AMPK catalytic subunit, phosphorylated AMPK T172 (P-AMPK), phosphorylated acetyl Coenzyme A carboxylase (P-ACC), and phosphorylated Akt (P-Akt) S473 and T308 were purchased from Cell Signaling Technology (Beverly, MA, USA). Antibodies against phosphorylated ATM S1981 (P-ATM) were from EMD Millipore (Billerica, MA, USA). Horseradish peroxidase-linked streptavidin and antibodies against ATM and tubulin were obtained from Sigma-Aldrich Corporation (St. Louis, MO, USA). Horseradish peroxidase-conjugated goat anti-rabbit and goat anti-mouse IgG were obtained from Pierce Biotechnology (Rockford, IL, USA). Chloroquine diphosphate (CQ), resveratrol (RE), and 1,2-Bis(2-aminophenoxy) ethane-N,N,N',N'-tetraacetic acid tetrakis acetoxymethyl ester (BAPTA-AM) were purchased from Sigma-Aldrich. Dr. Graeme Smith (KuDOS Pharmaceuticals, Cambridge, UK) generously

provided the ATM inhibitor KU55933 (KU). Calcium Green-1 AM was from Invitrogen Life Technologies (Grand Island, NY).

## 2.2. Cell culture

C2C12 myoblasts were obtained from the American Type Culture Collection (Rockville, MD, USA). C2C12 cells were cultured in Dulbecco's Minimum Essential Medium (DMEM) containing 10% FetalPlex (Gemini Bio-Products, Woodland, CA) and 1% penicillin/streptomycin. C2C12 cells were passaged by using a 5% trypsin, 0.02% EDTA solution every one to two days. Cells were differentiated for two days by incubating with DMEM containing 2% horse serum and 1% penicillin/streptomycin. Cells were grown in a humidified incubator with 5% CO<sub>2</sub> at 37 °C. For some experiments, myotubes were serum-starved by incubation with DMEM with antibiotic but without FetalPlex for three hours. C2C12 cell lines expressing green fluorescent protein (GFP) or short hairpin RNA to reduce ATM expression were described previously [5, 18].

## 2.3. Effects of CQ and resveratrol on phosphorylation of AMPK and Akt

Western blots were used to measure phosphorylation of AMPK and Akt after exposure to CQ and RE. Myotubes were incubated in DMEM containing 0 μM, 250 μM, 500 μM, or 750 μM CQ for 60 min. In separate experiments, myotubes were incubated in DMEM with 0 μM (vehicle: 0.1% DMSO), 50 μM, 100 μM, or 150 μM RE for one hour after a three hour serum starvation. After incubation, myotubes were placed on ice, media was aspirated, and cells were washed twice with phosphate-buffered saline. Cells were scraped in ice cold buffer containing 50 mM HEPES, pH 7.4, 2 mM Na<sub>3</sub>VO<sub>4</sub>, 150 mM NaF, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 0.5 μg/ml pepstatin and 1 mM phenylmethylsulfonyl fluoride. Cells were centrifuged at 14,000×g for 10 min, and supernatant was kept. Protein concentration of the supernatant was measured using a bicinchoninic assay from Thermo Scientific (Rockford, IL, USA).

To determine whether ATM plays a role in response to CQ and RE, C2C12 myotubes expressing shRNA against ATM or myotubes that were pretreated with the ATM inhibitor KU-55933 (KU) [24] were used. C2C12 cells expressing shRNA against ATM or GFP as a control were incubated in the presence or absence of 500 μM CQ for one hour. In separate experiments, C2C12 myotubes were pretreated for 30 min with either 10 μM or 5 μM KU-55933 (DMSO vehicle). Myotubes were then incubated in the absence or presence of 500 μM CQ or 150 μM RE with KU-55933 still present if it had been present in the pre-incubation. After an one hour exposure to CQ or RE, cells were lysed as described above.

## 2.4. Western blotting

Western blot procedures have been previously described [11]. Aliquots of cell lysates were diluted in Laemmli sample buffer containing dithiothreitol, boiled for 5 min, and separated on 4–20% Tris-HEPES-SDS polyacrylamide gels (Thermo Scientific) or 3–8% Tris-acetate gels (Life Technologies, Grand Island, NY, USA) alongside HiMark (Life Technologies) high molecular weight protein standards for analysis of ATM and P-ATM. After transfer to nitrocellulose and blocking in 5% nonfat dry milk in Tris-buffered saline, membranes were probed with the primary antibodies described in the Materials section, probed with secondary antibodies conjugated to horseradish peroxidase, and subjected to enhanced chemiluminescence analysis with Western Lightning reagents (Perkin Elmer, Waltham, MA, USA). ACC was detected with horseradish peroxidase conjugated to streptavidin, which binds the biotin moiety of ACC.

## 2.5. Glucose transport

Glucose transport assays were done as measured previously [5]. Myotubes were serum-starved for three hours before incubation in serum-free medium for 30 min in the presence or absence of 10  $\mu$ M KU55933. After the preliminary incubation with vehicle (DMSO) or KU55933, the myotubes were incubated for one hour with 0  $\mu$ M or 500  $\mu$ M CQ, with DMSO or KU55933 still present. Myotubes were then rinsed with glucose-free HEPES-buffered saline and then assayed for rates of 2-deoxyglucose (2DG). Glucose transport assays were performed by incubating cells with glucose-free HBS containing radiolabeled 2-DG (3  $\mu$ Ci/ml, American Radiolabeled Chemical, Inc., St. Louis, MO, USA) for 10 min at room temperature. 10  $\mu$ M cytochalasin B was used to measure GLUT independent glucose transport. After the 10 min incubation, the medium was aspirated, and the cells were rinsed twice with ice-cold saline. Cells were lysed in 0.2 N NaOH containing 0.2% SDS and  $^3$ H in samples was measured with a Tri-Carb 3110 TR Liquid Scintillation Analyzer (PerkinElmer, Waltham, MA). Glucose transport was normalized to protein concentrations of cell lysates measured by bicinchoninic acid (BCA) assay method (Pierce, Rockford, IL).

## 2.6. Intracellular calcium

Calcium measures were essentially as described for a plate reader-based intracellular calcium assay using Calcium Green-1 AM [25]. Myotubes in 96 well plates were incubated for 20 min at room temperature in phosphate buffered saline containing 5 mM glucose and 4  $\mu$ M Calcium Green-1 AM, a membrane permeable fluorophore that increases in fluorescence emission as  $[Ca^{2+}]$  increases. Cells were washed with PBS, and then cells were incubated in a humidified 5% CO<sub>2</sub> incubator at 37 °C for 20 min in DMEM containing

DMSO (vehicle) or 30  $\mu\text{M}$  BAPTA-AM, a membrane permeable  $\text{Ca}^{2+}$  chelator. Cells were washed with PBS and then incubated in the absence or presence of 0.5 mM CQ and in the presence or absence of BAPTA-AM for 60 min in HEPES-buffered saline (HBS: 5 mM glucose, 20 mM HEPES, 140 mM NaCl, 5 mM KCl, 2.5 mM  $\text{MgSO}_4$ , and 1 mM  $\text{CaCl}_2$ ). Fluorescence readings were taken with an excitation of 485 nm and emission of 590 nm and corrected for background fluorescence for cells treated identically except for preloading with Calcium Green-1 AM. In an additional experiment, cells were preloaded with Calcium Green-1 AM, incubated in the presence or absence of BAPTA-AM, and then exposed to CQ in calcium-free medium (PBS with 5 mM glucose).

## 2.7. Statistics

Data were analyzed by one-way ANOVA with *post hoc* Least Significant Difference (LSD) comparisons when  $P < 0.05$  for the ANOVA.

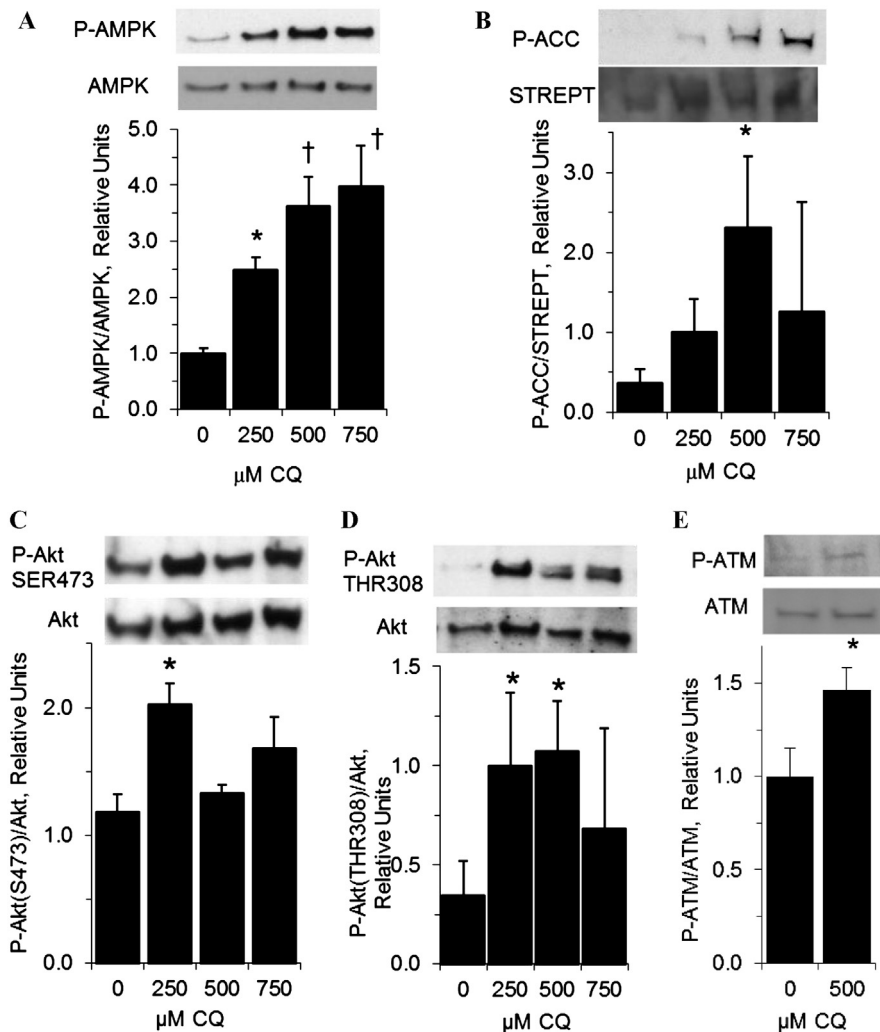
## 3. Results

### 3.1. Effects of chloroquine on AMPK and Akt

As it has been suggested that ATM is upstream of AMPK [12, 13, 14, 15, 16], we hypothesized that the ATM activator CQ [19] would stimulate AMPK phosphorylation. Fig. 1A shows that myotubes incubated in concentrations of 250 and 500  $\mu\text{M}$  CQ had a two and three fold increase of P-AMPK respectively, when compared to control cells ( $P < 0.05$  and  $P < 0.001$ ,  $n = 3/\text{group}$ ). There was no further increase in P-AMPK for 750  $\mu\text{M}$  CQ compared to 500  $\mu\text{M}$  CQ. A direct downstream target of AMPK is ACC, the phosphorylation of which was measured as a marker for increased AMPK activity. Fig. 1B illustrates that 500  $\mu\text{M}$  CQ caused better than a two fold increase in P-ACC compared to cells without CQ ( $P < 0.05$ ,  $n = 3/\text{group}$ ).

Phosphorylation of Akt was also measured since it has been reported that ATM plays a role in Akt activation by insulin or ionizing radiation [17]. Shown in Fig. 1C, exposure to 250  $\mu\text{M}$  CQ caused a two fold increase in P-Akt at Ser 473 compared to the control cells ( $P < 0.05$ ,  $n = 3/\text{group}$ ). Fig. 1D shows that 250  $\mu\text{M}$  CQ and 500  $\mu\text{M}$  CQ also caused an increase in P-Akt at Thr 308 ( $P < 0.05$ ,  $n = 3/\text{group}$ ). Phosphorylation of S1981, ATM's autophosphorylation site, was assessed as a marker for ATM activation. Fig. 2E shows that myotubes exposed to 500  $\mu\text{M}$  CQ had an increased amount of phosphorylated ATM compared to the control ( $P < 0.05$ ,  $n = 6/\text{group}$ ), indicating that CQ activates ATM in myotubes.

In summary, Fig. 1 provides evidence that CQ is an ATM activator and increases phosphorylation of AMPK, ACC, and Akt in C2C12 myotubes.

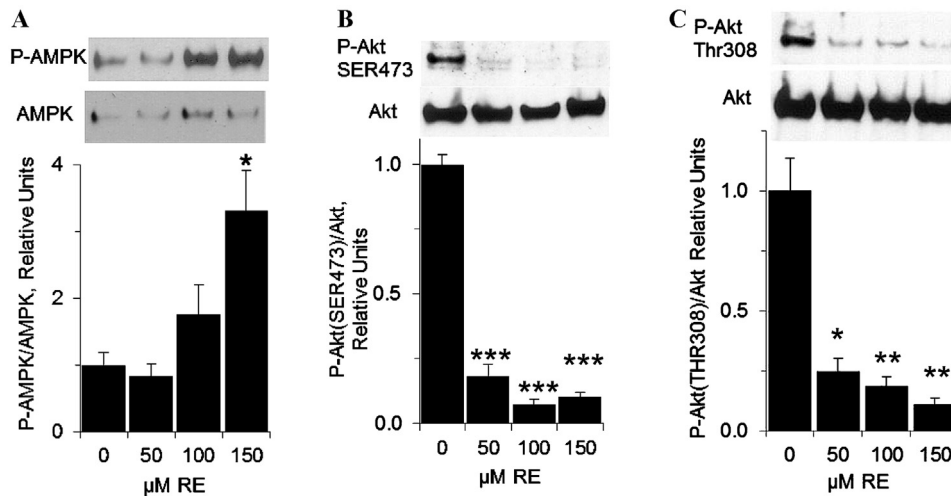


**Fig. 1. Chloroquine increases phosphorylation of AMPK and Akt in C2C12 myotubes.**

Myotubes were incubated in indicated concentrations of chloroquine (CQ) for one hour. Proteins were extracted and subjected to western blot with specific antibodies for (A) phosphorylated AMPK (P-AMPK) normalized to AMPK, (B) P-ACC, normalized to streptavidin, (C) P-Akt S473 normalized to Akt, (D) P-Akt T308 normalized to Akt, and (E) P-ATM normalized to tubulin. Streptavidin was used to detect the biotin moiety of ACC. Representative blots are shown. Data are means  $\pm$  SE; Symbols indicate statistical difference from control not exposed to CQ: \* $P < 0.05$ , † $P < 0.001$ ;  $n = 3$ /group for panels A–D and  $n = 6$ /group for panel E. Full images of western blots for Fig. 1 are available in the supplementary material as supplementary Figs. 1A–E.

### 3.2. Effects of resveratrol

We then used another reported ATM activator, RE, to examine whether results shown above were specific to CQ or could be generalized to other ATM activators. Fig. 2A shows that 150  $\mu$ M RE increased phosphorylation of AMPK compared to vehicle treated controls ( $P < 0.05$ ,  $n = 3$ /group). Phosphorylation of Akt at Thr 308 and Ser 473 in response to RE was also measured. Panels 2B



**Fig. 2. Resveratrol stimulates phosphorylation of AMPK but not Akt.** Myotubes were incubated with either DMSO (vehicle) or the indicated concentration of RE for one hour and analyzed by Western blot. Blots were probed for (A) P-AMPK normalized to AMPK, (B) P-Akt (SER473) normalized to Akt, and (C) P-Akt (THR308) normalized to Akt. Representative blots are shown. Data represent means  $\pm$  SE. Symbols indicate statistically significant difference from control; \* $P < 0.05$ , \*\* $P < 0.005$ , \*\*\* $P < 0.0005$ ;  $n = 3/\text{group}$  for all panels. Full images of western blots for Fig. 2 are available in the supplementary material as supplementary Figs. 2A–C.

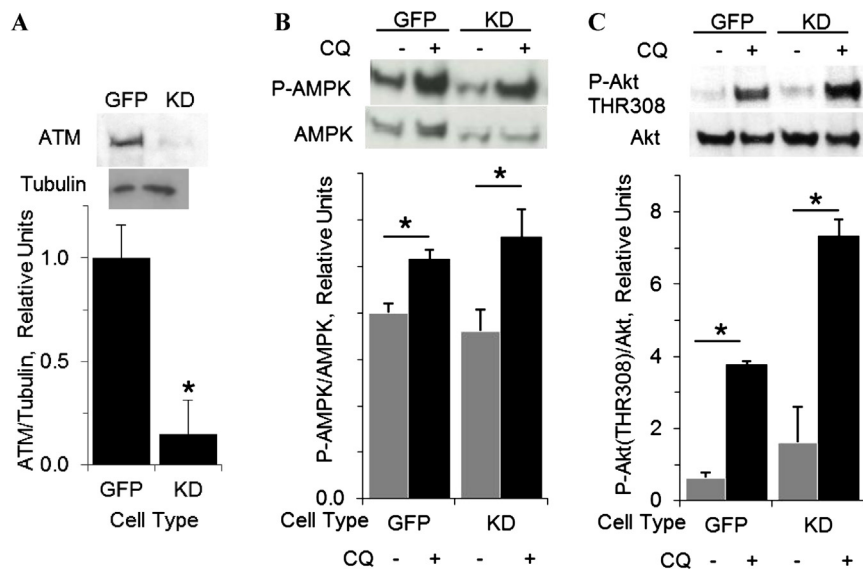
and 2C illustrate that RE treatment does not increase phosphorylation of Akt at Ser 473 or T308 ( $n = 3/\text{group}$ ), suggesting that incubation of myotubes with a purported ATM activator, RE, is not sufficient to stimulate phosphorylation of Akt. RE treatment not only did not increase Akt phosphorylation, but caused its inhibition ( $P < 0.05$ ,  $n = 3/\text{group}$ ). This phenomenon has been reported previously, though not in myotubes to our knowledge [26, 27].

### 3.3. Role of ATM in CQ and RE effects

To examine the role that ATM plays in CQ-stimulated phosphorylation of AMPK and Akt, we used C2C12 cell lines that expressed either shRNA against ATM (expression knocked down, KD) or green fluorescent protein (GFP). Fig. 3A shows that the KD cell line had an 80% decrease in ATM protein levels compared to protein levels in cell lines transduced with GFP ( $P < 0.05$ ,  $n = 4/\text{group}$ ). Fig. 3B shows that even in the KD cell lines, 500  $\mu\text{M}$  CQ stimulated phosphorylation of AMPK ( $P < 0.05$ ,  $n = 5/\text{group}$ ). The shRNA against ATM also did not have an effect on CQ stimulated phosphorylation of Akt at Thr 308, as shown in Fig. 3C. ( $P < 0.05$ ,  $n = 3/\text{group}$ ). In summary, Fig. 3 shows that a decrease of ATM protein does not have an effect on CQ-stimulated phosphorylation of AMPK and Akt.

To confirm results that were shown with the KD cell line, a specific ATM inhibitor was used, KU55933 (KU), that has an  $\text{IC}_{50}$  concentration of 13 nM

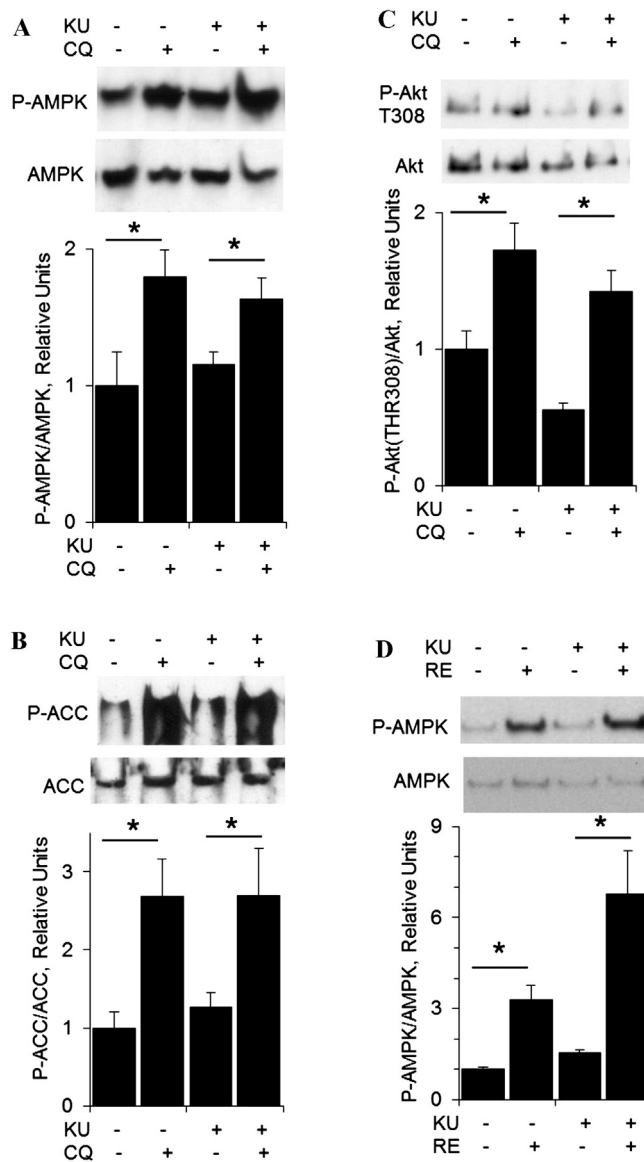




**Fig. 3. Chloroquine stimulates phosphorylation of AMPK and Akt in ATM deficient cells.** To assess whether CQ's effects were dependent on ATM, a C2C12 cell line was used that expressed shRNA against ATM (expression knocked down, KD). C2C12 myotubes transduced with green fluorescent protein (GFP) was used as a control. GFP and KD myotubes were incubated in indicated concentrations of CQ for one hour. Proteins were extracted and subjected to Western blot. Blots were probed for (A) ATM normalized to tubulin, (B) P-AMPK normalized to AMPK, and (C) P-Akt T308 normalized to Akt. Representative blots are shown. Data are means  $\pm$  SE.  $n = 4$ /group for panel A,  $n = 4$ /group for panel B, and  $n = 3$ /group for panel C. \*indicates statistically difference,  $P < 0.05$ , from GFP-expressing cells (panel A) or different from corresponding cell line not treated with CQ (panels B and C). Full images of western blots for Fig. 3 are available in the supplementary material as supplementary Figs. 3A–C.

[24]. We used the compound at 10  $\mu$ M, because previous research has shown that this concentration was sufficient to inhibit ATM [28]. In our hands, 1  $\mu$ M KU is also sufficient to prevent ATM action in myotubes [11]. Pretreatment with 10  $\mu$ M KU did not prevent CQ stimulated phosphorylation of AMPK, shown in Fig. 4A. ( $P < 0.05$ ,  $n = 3$ /group). The addition of KU did not inhibit the phosphorylation of the downstream target of AMPK, ACC ( $P < 0.01$ ,  $n = 3$ /group), as shown in Fig. 4B. Fig. 4C displays that KU did not interfere with CQ stimulated phosphorylation of Akt at Thr 308 ( $P < 0.05$ ,  $n = 3$ /group). As shown in Fig. 4D, 5  $\mu$ M KU had no effect on the increased phosphorylation of AMPK stimulated by RE ( $P < 0.05$ ,  $n = 3$ /group). In summary, Fig. 4 demonstrates that the presence of ATM inhibitor, KU, does not have an effect on CQ and RE stimulated phosphorylation of AMPK or CQ stimulated phosphorylation of Akt.

Taken together, these results in Fig. 3 and Fig. 4 suggest that stimulation of AMPK and Akt by CQ is ATM-independent in C2C12 myotubes.



**Fig. 4. Chloroquine and resveratrol stimulate phosphorylation of AMPK and chloroquine stimulates phosphorylation of Akt when ATM is inhibited.** C2C12 myotubes were placed in medium containing either 10  $\mu$ M KU or vehicle (DMSO) for 30 min. 500  $\mu$ M CQ (CQ) was added to both KU- and DMSO-incubated cells. C2C12 lysates were analyzed by Western blot and probed for (A) P-AMPK normalized to AMPK, (B) P-ACC normalized to ACC, (C) and P-Akt (THR308) normalized to Akt. (B) After serum starvation, myotubes were placed in medium containing either 5  $\mu$ M KU or 1 vehicle (DMSO) for thirty minutes. Myotubes were then incubated with either DMSO (vehicle) or 150  $\mu$ M RE for one hour. Myotubes were lysed and probed for P-AMPK normalized to AMPK. Representative blots are shown. Data are means  $\pm$  SE; \* indicates a statistically different value from corresponding cells not exposed to CQ,  $P < 0.05$ .  $n = 3$ /group for all panels. Full images of western blots for Fig. 4 are available in the supplementary material as supplementary Figs. 4A–D.

### 3.4. Effect of CQ on glucose uptake

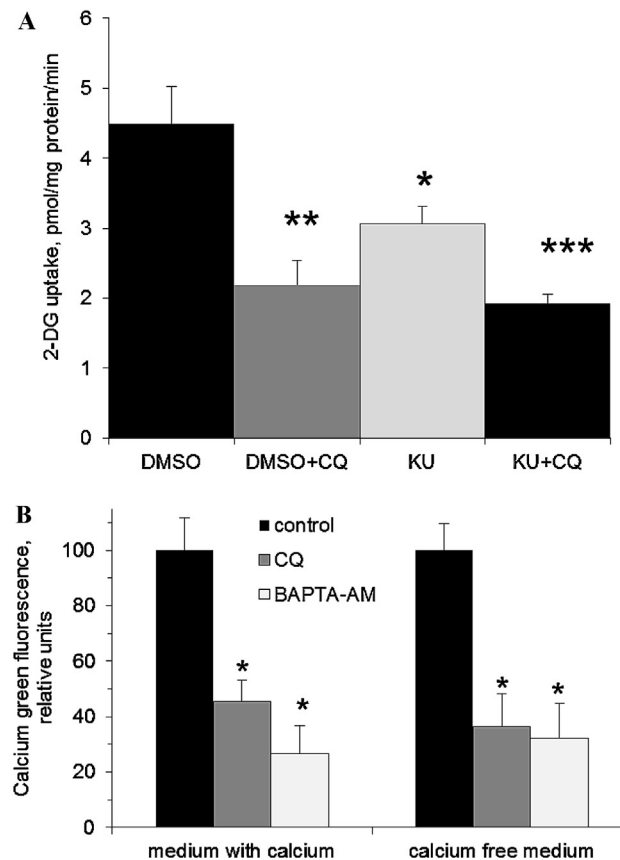
CQ's effect on glucose uptake in C2C12 myotubes was also measured. While the long term effects of CQ improving whole body metabolism is dogma [20, 21, 29, 30], research on CQ's acute effects in cell culture have produced conflicting data. It has been reported that 100  $\mu\text{M}$  CQ increases glucose transport in myotubes [31]. However, other papers have shown that 200  $\mu\text{M}$  CQ inhibits insulin-stimulated GLUT4 translocation to the in cell membrane in fat cells, and three hour CQ treatment had no effect on glucose uptake in human forearm muscle [32, 33]. Other researchers have shown that CQ has no effect on insulin-stimulated glucose transport in adipocytes [34]. In our hands, 500  $\mu\text{M}$  CQ did not cause an increase in basal glucose transport and actually slightly inhibited it (Fig. 5A,  $P < 0.005$ ,  $n = 9-10/\text{group}$ ). The ATM inhibitor KU also inhibited glucose transport, a finding we have reported previously [3].

### 3.5. Effect of chloroquine on intracellular $[\text{Ca}^{2+}]$

Recently, CQ has been identified as a ligand for MAS-related G protein coupled receptors (Mrgpr), including family members A3, A1, and X1 [35]. Binding of CQ to these receptors activates  $\text{Ca}^{2+}$  flow via transient receptor potential (TRP) cation channels and also activates phospholipase C activity that would stimulate IP3-mediated  $\text{Ca}^{2+}$  from intracellular calcium stores. At least two Mrgpr-responsive TRP cation channels, TRPA1 and TRPC3 are expressed in mouse skeletal muscle [36]. Increases in cytosolic calcium levels have been shown to activate Akt in myotubes [37, 38, 39], and there are  $\text{Ca}^{2+}$ -dependent pathways for activation of AMPK [40]. Accordingly, we assessed intracellular  $\text{Ca}^{2+}$  using Calcium Green-1 AM, a membrane permeable fluorophore with fluorescence emission increasing as  $\text{Ca}^{2+}$  concentration increases. As shown in Fig. 5, CQ actually decreased intracellular calcium to the level achieved with BAPTA-AM, a membrane permeable  $\text{Ca}^{2+}$  chelator. This reduction of intracellular  $\text{Ca}^{2+}$  by CQ occurs even in  $\text{Ca}^{2+}$ -free medium, suggesting that CQ decreases  $\text{Ca}^{2+}$  release from intracellular  $\text{Ca}^{2+}$  stores, such as the sarcoplasmic reticulum or lysosomes. Because CQ decreases intracellular  $\text{Ca}^{2+}$  – which is opposite from what would be expected if CQ were to act through a Mrgpr – a receptor mediated role of CQ in the activation of AMPK and Akt in myotubes seems unlikely. It seems likely that in our hands CQ inhibits glucose uptake by decreasing the abundance of intracellular calcium. This is consistent with a previous finding that BAPTA-AM suppresses glucose transport [41].

## 4. Discussion

Both CQ and RE are reported ATM activators [19, 42]. However, their roles in phosphorylation of AMPK have not been previously addressed in skeletal



**Fig. 5. Chloroquine does not increase glucose transport but decreases intracellular  $[Ca^{2+}]$ .** (A) Glucose transport was measured for 10 min in C2C12 myotubes. Both 1 hour of 0.5 mM CQ as well as 10  $\mu$ M KU caused a decrease in glucose transport compared to the DMSO alone treatment. (B) Myotubes were preloaded with the fluorescent calcium sensor Calcium Green-1 AM and then incubated in the absence or presence of BAPTA-AM, a cell permeable calcium chelator, before exposure to 0.5 mM CQ for one hour. As indicated by Calcium Green fluorescence, CQ decreased intracellular  $Ca^{2+}$  to about the same level as BAPTA-AM both in medium containing  $Ca^{2+}$  (left) and  $Ca^{2+}$ -free medium (right). Symbols indicate a significant difference from control, \* $P < 0.05$ , \*\* $P < 0.005$ , \*\*\* $P < 0.0005$ ;  $n = 9-12$  per group.

muscle cells. This study provides evidence that CQ and RE increase phosphorylation of AMPK independent of ATM in C2C12 myotubes. Consistent with this, ATM did not play a role in CQ-stimulated phosphorylation of AMPK's downstream target, ACC. This study also showed that CQ increases phosphorylation of Akt at S473 and T308 independent of ATM. The role of ATM was assessed by using either the ATM specific inhibitor, KU [24] or C2C12 cells expressing shRNA against ATM. Regardless of ATM inhibition or ATM deficiency, incubation with CQ increased phosphorylation of AMPK and Akt. Thus, CQ acts independently of ATM to activate AMPK and Akt in myotubes. An additional, novel aspect of the study is the demonstration that CQ decreases intracellular calcium concentration, probably through preventing  $Ca^{2+}$

from intracellular stores. This decreased intracellular calcium possibly explains the failure of CQ to stimulate glucose transport.

The AMP-activated protein kinase (AMPK) is a heterotrimeric kinase that marshals responses to metabolic stress [40]. Downstream actions of AMPK include insulin-independent glucose disposal, increased insulin action, increased fat oxidation, and decreased fatty acid synthesis. Activation of AMPK with metformin before ischemia or during reperfusion in mice is cardioprotective, as demonstrated by substantial decrease in infarct size and preservation of ejection fraction, perhaps through AMPK's activation of endothelial nitric oxide synthase (eNOS) [43]. The pre-ischemia protective effect of metformin was greater than the effect of metformin during reperfusion, suggesting that chronic treatment with an AMPK-activating compound could have cardioprotective value. In rats, activation of AMPK with Abbot Compound A769662 during ischemia decreased infarct size associated with increased glycogen synthase kinase 3 $\beta$  (GSK3 $\beta$ ) phosphorylation [44].

A few earlier studies reported that ATM is an upstream activator of AMPK [13, 45]. For example, AMPK phosphorylation in response to insulin-like growth factor 1 (IGF1) was decreased in ATM-deficient HeLa cells [12]. Additional evidence suggests that ATM is required for 5-Aminoimidazole-4-carboxamide ribonucleotide (AICAR)-stimulated phosphorylation of AMPK [13]. AICAR-stimulated phosphorylation of AMPK was blunted in ATM-deficient mouse embryonic fibroblasts or in the presence of the ATM inhibitor KU. AICAR-stimulated phosphorylation of AMPK was also inhibited in human HeLa cells in the presence of KU [13].

Our data suggest that there is a mechanism other than activation of ATM through which CQ and RE increase phosphorylation of AMPK. AMPK is activated by an increase in the AMP:ATP ratio [40]. Consistent with this idea, it has been shown that CQ inhibits mitochondrial proteins, such as cytochrome c oxidase, which would lead to a decrease in ATP production [46]. CQ has also been reported to be an uncoupling agent [47], and uncoupling of respiration from ATP synthesis could be a means by which CQ induces metabolic stress. Notably, in regard to the discussion of Akt that follows, mild uncoupling by use of dinitrophenol has been reported to increase Akt phosphorylation three fold in skeletal muscle [47]. RE exposure might also lead to decreased ATP production. For example, previous research has shown that RE inhibits ATP synthase [48]. Thus, it seems probable that CQ and RE could both activate AMPK by causing metabolic stress. If so, this would be a mechanism shared by a number of AMPK-activating compounds, such as metformin, quercetin, and berberine [40].

In addition to possibly inhibiting ATP production, our findings suggest that at higher concentrations, CQ and RE inhibit Akt. It could simply be that an

increase of AMPK activation could lead to direct inhibition of Akt. This finding that AMPK agonists regulate Akt activation has been reported previously. Phenformin as well as AICAR both increased AMPK phosphorylation while decreasing Akt phosphorylation in neuroblastoma cells [49]. The AMPK agonist metformin also inhibits Akt phosphorylation in cervical cancer cells [50]. Our results and previous reports [51, 52, 53] have shown that RE does not increase phosphorylation of Akt and actually inhibits it. Other labs have also discovered this finding of RE inhibiting Akt phosphorylation in both breast cancer and liver cell lines [54, 55]. A possible explanation of how RE inhibits Akt is its effect of inhibiting cAMP phosphodiesterases [52]. Inhibition of phosphodiesterases leads to an increase in cAMP, which would inhibit localization of PDK1 at the plasma membrane, thus decreasing Akt activation [56].

Akt plays a central role in regulation of glucose transport in insulin-responsive tissues [57] and is a key positive regulator of glycogen synthesis through inhibition of glycogen synthase kinase 3 $\beta$  (GSK3 $\beta$ ). In cardiomyocytes, GSK3 $\beta$  with a mutation preventing its activation by Akt is protective against cardiac hypertrophy and suppresses the protective effects of ischemic preconditioning [58]. In some tissue or cell types, ATM appears to be an upstream activator of Akt. For example, activating phosphorylation of Akt in response to insulin signaling is ATM dependent in human fibroblast cells [17], and IGF-1 failed to stimulate Akt phosphorylation in cultured myotubes when ATM was inhibited or knocked down with shRNA [18]. Likewise, Akt phosphorylation was reduced in the liver and aorta of mice heterozygous for a truncation mutation of ATM gene [30]. Finally, insulin-stimulated phosphorylation of Akt was blunted in fast twitch muscle of mice deficient in ATM [6], and ATM was reportedly required for full activation of Akt by insulin in cultured muscle cells [7]. Taken together, the reported roles of ATM upstream of Akt make activation of ATM an attractive strategy for improving a number of factors relevant to cardiovascular disease, including glucoregulation and cardioprotection.

On the surface, it is surprising that CQ stimulation of AMPK and Akt phosphorylation did not result in an increase in glucose transport. However, when taken in the light of CQ's effects on intracellular calcium, this makes sense. Multiple labs have shown that chelation of intracellular calcium actually blocks insulin action in adipocytes and fibroblasts [41, 59, 60]. It has also been reported that basal glucose transport through GLUT1 requires cytosolic calcium ions in rat epithelial cells [61]. Thus, the decrease in intracellular calcium in the presence of CQ is a likely explanation for the lack of positive effect of CQ on glucose transport.

Given its ability to activate ATM, AMPK, and Akt—which together play prominent roles in responsive to genotoxic, oxidative, and metabolic stress, are

central regulators of fuel uptake, storage, and oxidation, and play key cardioprotective roles—CQ can exert positive metabolic effects, even in cell types in which ATM does not activate AMPK or Akt. The additional novel finding that CQ decreases intracellular calcium levels suggests that CQ could have important calcium-dependent vascular effects. For example, increased intracellular calcium has been linked to increased vasoconstriction and heart failure [62, 63]. In summary, cellular effects of CQ and RE are not limited to their activation of ATM. CQ especially can serve as an example compound able to modify disparate metabolic regulators, including ATM, AMPK, Akt, and intracellular calcium.

## 5. Conclusions

In summary, we have demonstrated that the ATM activator chloroquine stimulates AMPK and Akt phosphorylation in a manner that does not require ATM. Likewise, stimulation of Akt phosphorylation by the ATM activator resveratrol is independent of ATM. We have also shown that chloroquine causes a decrease in cytosolic  $\text{Ca}^{2+}$  concentration. Thus, we have uncovered multiple chloroquine effects that are separate from chloroquine's activation of ATM.

## Declarations

### Author contribution statement

Larry D. Spears, Jonathan S. Fisher: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Andrew V. Tran, Charles Y. Qin, Supriya B. Hobbs, Cheryl A. Liang Burns, Nathaniel K. Royer: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data.

Zhihong Zhang: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data.

Lyle Ralston: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

### Conflict of interest statement

The authors declare no conflict of interest.

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