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Chloroquine stimulates glucose uptake and glycogen synthase in muscle cells through activation of Akt

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ABSTRACT

Chloroquine is a pharmaceutical agent that has been widely used to treat patients with malaria. Chloroquine has also been reported to have hypoglycemic effects on humans and animal models of diabetes. Despite many previous studies, the mechanism responsible for its hypoglycemic effect is still unclear. Chloroquine was recently reported to be an activator of ATM, the protein deficient in the Ataxia-telagiectasia (A-T) disease. Since ATM is also known as an insulin responsive protein that mediates Akt activation, we tested the effect of chloroquine on the activity of Akt and its downstream targets. In L6 muscle cells treated with insulin and chloroquine, the phosphorylation of Akt and glucose uptake were dramatically increased compared to cells treated with insulin alone, suggesting that chloroquine is a potent activator of Akt and glucose uptake in these cells. We also found that the reduction of insulin-mediated Akt activity in muscle tissues of insulin resistant rats was partially reversed by chloroquine treatment. Moreover, insulin-mediated phosphorylation of glycogen synthase kinase-3β in L6 cells was greatly enhanced by chloroquine. A substantial decrease in phosphorylation of glycogen synthase was also observed in chloroquine-treated L6 cells, indicating enhanced activity of glycogen synthase. Taken together, our results not only show that chloroquine is a novel activator of Akt that stimulates glucose uptake and glycogen synthase, but also validate chloroquine as a potential therapeutic agent for patients with type 2 diabetes mellitus.

1. Introduction

Chloroquine has long been used in clinical settings for treating patients with malaria. Chloroquine has also been tested in multiple animal models of type 2 diabetes (T2D) and has led to significant improvement of insulin sensitivity and glucose tolerance [1,2]. Despite intensive studies involving its role in glucose metabolism, the underlying mechanism of chloroquine's glucose regulatory function remains elusive.

Recently, chloroquine was shown to activate ATM, a protein kinase deficient in Ataxia-telangiectasia (A-T) disease [3]. Another recent study investigated the effect of chloroquine on insulin resistance in mice with ATM deficiency and an Apolipoprotein (ApoE) null background. Results showed that chloroquine increases glucose tolerance in $ATM^{+/+}ApoE^{-/-}$ mice fed with a western (high-fat) diet but not in $ATM^{-/-}ApoE^{-/-}$ mice fed with the same diet, indicating the effect of chloroquine on glucose tolerance is ATM-dependent [4].

A-T is a rare autosomal recessive inherited disease mainly characterized by progressive ataxia and oculocutaneous telangiectasias [5,6]. In addition, A-T disease is also associated with an increased incidence of T2D mellitus. A-T patients with T2D exhibit symptoms of insulin resistance and glucose intolerance [7,8]. The gene mutated in this disease, *ATM* (A-T, mutated), encodes a 370-kDa protein kinase. Traditionally, ATM was considered a nuclear protein, which controls cell cycle progression in response to genotoxic stress [5,6]. Yet, it is difficult to explain many of the growth-related abnormalities of A-T, such as growth retardation and insulin resistance, by the nuclear localization and function (i.e. DNA damage sensor) of ATM.

It is now known that ATM is also present in the cytoplasm and has separate cytoplasmic functions [9,10]. Important clues about the cytoplasmic functions of ATM have come from the discovery of its involvement in distinct insulin signaling pathways. ATM kinase activity was found to increase dramatically in response to insulin in rat 3T3-L1 cells that had differentiated into adipocytes [10]. The same study also demonstrated that ATM promotes protein translation initiation by phosphorylating an insulin responsive cytoplasmic protein, 4E-BP1.

More recently, it was discovered that ATM activates Akt by stimulating its phosphorylation following insulin treatment [11–14]. Akt is a major component in insulin signaling pathways and has been shown to participate in multiple physiological processes, including glucose uptake, cell growth, and cell survival.

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Therefore, the discovery of the connection between ATM and Akt could provide explanations for many of the clinical phenotypes of A-T that cannot be explained by the nuclear functions of ATM [15].

Insulin resistance is a hallmark of T2D. Studies have shown that disruption of insulin-mediated glucose transport is the major underlying cause of insulin resistance. Insulin-mediated glucose uptake occurs mainly in skeletal muscle tissue [16], which plays a crucial role in regulating glucose homeostasis. Akt is a central regulator of glucose uptake in muscle cells [17]. Our recent studies have shown that ATM facilitates insulin-mediated glucose uptake in muscle cells by stimulating Akt activity and the translocation of glucose transporter 4 (GLUT4) from cytoplasm to the cell surface [11].

In this study, we tested the effect of chloroquine on Akt activity in L6 rat muscle cells and in muscle tissues of high-fat fed rats that have developed insulin resistance. We also examined the effect of chloroquine on glucose uptake in L6 cells. Moreover, we analyzed chloroquine's effect on the phosphorylation of glycogen synthase kinase-3β (GSK-3β) and glycogen synthase (GS). Our studies suggest that chloroquine is not only a novel activator of Akt, but also a stimulator of multiple physiological events downstream of Akt, including glucose uptake and glycogen synthesis in muscle cells.

2. Materials and methods

2.1. Materials

The anti-β-actin and anti-β-tubulin antibodies were from Sigma. Antibodies against GSK-3β(Ser-9), phospho-Akt(Ser473), phospho-JNK(Thr183/Tyr185), and phospho-GS(Ser641) were from Cell Signaling. ^{3}H-2-deoxyglucose (2-DG) was purchased from Perkin Elmer. Insulin, chloroquine, and caffeine were purchased from Sigma, and KU-55933 was from Calbiochem.

2.2. Chloroquine treatment of rats with insulin resistance

Male Wistar rats (Harlan) were used in the experiment starting at 3–4 weeks of age. Insulin resistance was induced in the rats through the feeding of a high-fat diet (35% lard by mass, Harlan Teklad) as previously described [11]. Control rats were given standard rodent chow (Harlan Teklad). After 3 months on the high-fat diet, rats were treated with 3.5 mg chloroquine/kg bodyweight through IP (intraperitoneally) injection, twice per week, for one month. Control rats were IP injected with saline for the same period of time.

2.3. Muscle tissue collection and homogenization following in vivo muscle insulin stimulation

After treatment with chloroquine, both high-fat fed rats and control rats were fasted overnight and then were IP injected with 20 units of insulin (Humulin R, Eli Lilly) 20 min prior to the start of continuous isoflurane anesthesia. Thirty minutes after the injection of insulin, the gastrocnemius muscle was excised from the animals. All muscle tissue was snap-frozen in liquid nitrogen. Frozen muscle tissue was later ground and homogenized in homogenization buffer as previously described [11].

2.4. Cell culture and preparation of cell lysate

Rat L6 myoblasts were grown in Modified Eagle's Medium-α (MEM-α) medium supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% fetal bovine serum. After treatments, subconfluent L6 cells were washed with cold phosphate buffered saline and then lysed on ice for 45 min using TGN lysis buffer [11]. Cell lysates were then centrifuged, and protein concentration of the supernatant was measured by the Lowry method.

2.5. SDS–PAGE and immunoblotting

Equal amounts of protein from cell lysates or rat muscle homogenates were subjected to SDS–PAGE. Western blotting was performed by transferring the proteins in the SDS–PAGE gel to a nitrocellulose or PVDF membrane. Immunoblotting was then carried out with antibodies against various proteins, and immunoreactive bands were visualized by chemiluminescence.

2.6. 2-DG incorporation analysis

The experiment was carried out as previously described [11]. Briefly, L6 muscle cells were rinsed with HEPES buffer and then incubated with a transport solution containing 10 μM 2-DG and 0.5 μCi/ml ^{3}H-2-DG for 5 min. Following 2-DG uptake, cells were washed 3 times with 0.9% NaCl and lysed with 0.05 N NaOH. The amount of ^{3}H-2-DG in cell lysates was determined using a scintillation counter. 2-DG uptake is measured as pmol of deoxyglucose/mg of protein/minute. Statistical significances of the results were analyzed by a Student's unpaired *t*-test.

3. Results

3.1. Chloroquine stimulates insulin-mediated Akt phosphorylation and glucose uptake in L6 muscle cells

Our previous studies indicate that inhibition of ATM in L6 muscle cells by its specific inhibitor, KU-55933, resulted in an abrogation of Akt phosphorylation at Ser 473 and a dramatic reduction of insulin-mediated glucose uptake [11]. Since chloroquine has recently been demonstrated as an activator of ATM [3], we tested whether chloroquine has a stimulatory effect on Akt and glucose uptake, opposite of what is observed with the ATM inhibitor.

Our results reveal that treatment of L6 myoblasts with chloroquine resulted in a dramatic increase of Akt phosphorylation at Ser473 in comparison to cells treated with insulin alone (Fig. 1A). In addition to confirming the role of ATM in the activation of Akt, this result also demonstrates that chloroquine is a novel activator of Akt in muscle cells. We then carried out a 2-deoxyglucose assay to determine the effect of chloroquine on glucose uptake in L6 cells. We found that glucose uptake was increased significantly in insulin-treated cells when compared to untreated control cells. Furthermore, in cells treated with both insulin and chloroquine, glucose uptake was 1.5-fold higher than what was observed in cells treated with insulin alone (Fig. 1B). Interestingly, glucose uptake in cells treated with chloroquine alone was also higher than that of untreated control cells. These results not only show that chloroquine is a potent activator of insulin-mediated glucose uptake in L6 muscle cells, but also suggest that it can mimic the effect of insulin on glucose uptake in these cells, even in the absence of insulin.

3.2. Chloroquine partially restores Akt phosphorylation at Ser473 in muscle tissue of high-fat fed rats

Muscle tissue from rats with insulin resistance is known to have decreased Akt phosphorylation at Ser473 compared to that of regular chow fed controls [11]. To further investigate the effect of chloroquine on insulin-mediated Akt activity *in vivo*, we induced insulin resistance in rats by feeding them a high-fat diet [11]. We

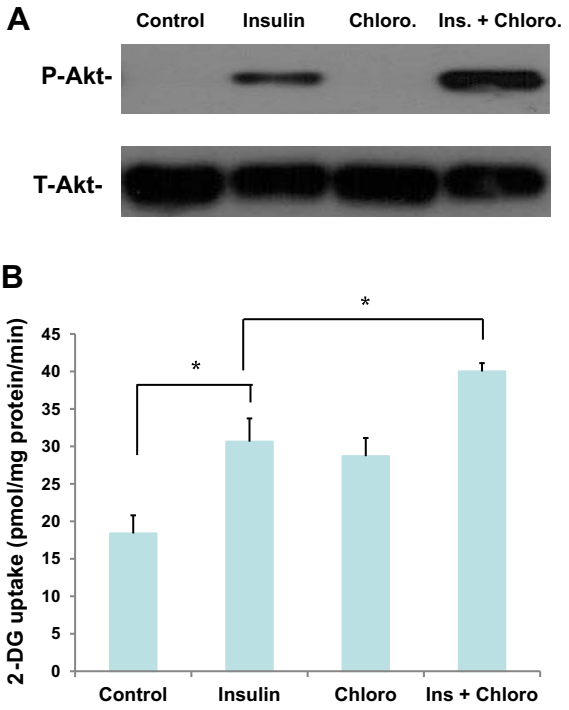

Fig. 1. A. Chloroquine stimulates Akt phosphorylation following insulin treatment in L6 cells. Subconfluent L6 myoblasts were serum-starved overnight and then treated with 100 μM chloroquine for 3 h before addition of 100 nM insulin for 45 min. Cells were then lysed and samples were subjected to SDS–PAGE. Levels of phospho-Akt(Ser473) and total Akt were determined by immunoblotting. The results are representative of 3 separate experiments. B. Chloroquine stimulates insulin-mediated glucose uptake in L6 cells. L6 myoblasts were serum-starved for 6 h before incubated with chloroquine followed by addition of insulin as described above. Following treatment, a 2-DG incorporation assay was performed to determine glucose uptake as described in Materials and Methods. The bar graph indicates the averages ± SEM of the 2-DG uptake values from 3 separate experiments ($^*p < 0.05$).

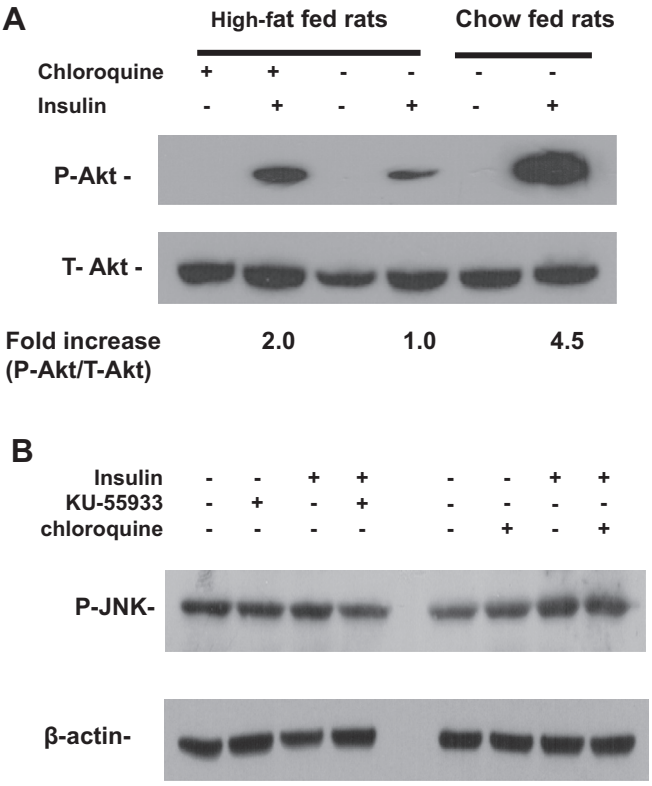

Fig. 2. A. Chloroquine partially restores Akt phosphorylation in muscle tissue of high-fat fed rats. Rats were fed either a high-fat diet or a chow diet. Rats on high-fat diets were injected with either saline or 3.5 mg of chloroquine/kg body weight twice per week. After one month of chloroquine treatment, the rats were fasted overnight and then injected with 20 units of insulin or an equal amount of saline solution. Rats were then anesthetized with isofluorane and the gastrocnemius muscle was removed. The muscle tissue was homogenized and subjected to SDS–PAGE. Phospho-Akt and total Akt levels were detected by Western blotting. B. Effect of chloroquine and KU-55933 on JNK phosphorylation in L6 cells treated with insulin. Subconfluent L6 myoblasts were serum-starved overnight and then treated with KU-55933 (10 μM) for 1 h or chloroquine (100 μM) for 3 h before the addition of 100 nM insulin for another 45 min. Cells were then lysed and protein samples were subjected to SDS–PAGE and Western blotting. The membranes were incubated with antibodies against phospho-JNK (Thr183/Tyr185 of p46 subunit of JNK) and β-actin, respectively. The results presented in A and B are representative of 3 separate experiments.

then proceeded to treat the high-fat fed rats with a low dose of chloroquine, and the effect of chloroquine on Akt activity in muscle tissue was examined.

A dramatic decrease in phospho-Akt at Ser473 was observed in high-fat fed rats when compared to chow fed rats. Treatment with chloroquine resulted in a substantial increase in levels of phospho-Akt at Ser473 in muscle tissue of high-fat fed rats versus vehicle treated high-fat fed rats. Thus, the inhibition of insulin-mediated Akt activity in muscle tissue of high-fat fed rats was partially reversed when these rats were treated with chloroquine (Fig. 2A).

3.3. Treatment with chloroquine and KU-55933 does not cause significant change in JNK phosphorylation in L6 cells treated with insulin

Results from Schneider et al. suggest that choloroquine may decrease JNK activity in an ATM-dependent manner [4]. However, recent studies have shown that while ATM levels decrease in skeletal muscle tissue of high-fat fed insulin resistant rats versus chow fed control rats, JNK activity remains similar [11]. Therefore, the role of ATM in the regulation of JNK activity is still controversial. In this study, we further tested the effect of chloroquine and KU-55933 on JNK phosphorylation in L6 muscle cells. As shown in Fig. 2B, JNK phosphorylation was not substantially altered by treatment with chloroquine or KU-55933, suggesting that JNK activity is not linked to changes of ATM activity.

3.4. Chloroquine stimulates while KU-55933 inhibits GSK-3β phosphorylation in L6 cells treated with insulin

Like glucose uptake, the conversion of glucose to glycogen by GS is also considered a rate-limiting step of glucose metabolism in muscle cells [18]. Glycogen synthesis is known to be controlled by Akt through its regulation of GSK-3 activity [19]. In quiescent condition, GSK-3 is constitutively active. Akt phosphorylates GSK-3 in response to insulin. In contrast to many other substrates of Akt, this phosphorylation event results in deactivation of GSK-3. We first examined the effect of KU-55933 on phosphorylation of GSK-3. KU-55933 inhibited phosphorylation of GSK-3β in the presence of insulin (Fig. 3A). By contrast, chloroquine caused a dramatic increase in insulin-mediated phosphorylation of GSK-3β (Fig. 3B), which suggests that chloroquine acts as a strong inhibitor of GSK-3β.

3.5. Chloroquine stimulates GS activity in L6 cells treated with insulin

Activated GSK-3 phosphorylates GS to inhibit its activity [18]. Deactivation of GSK-3 by insulin allows for dephosphorylation and activation of GS by its phosphatase. Activated GS will then

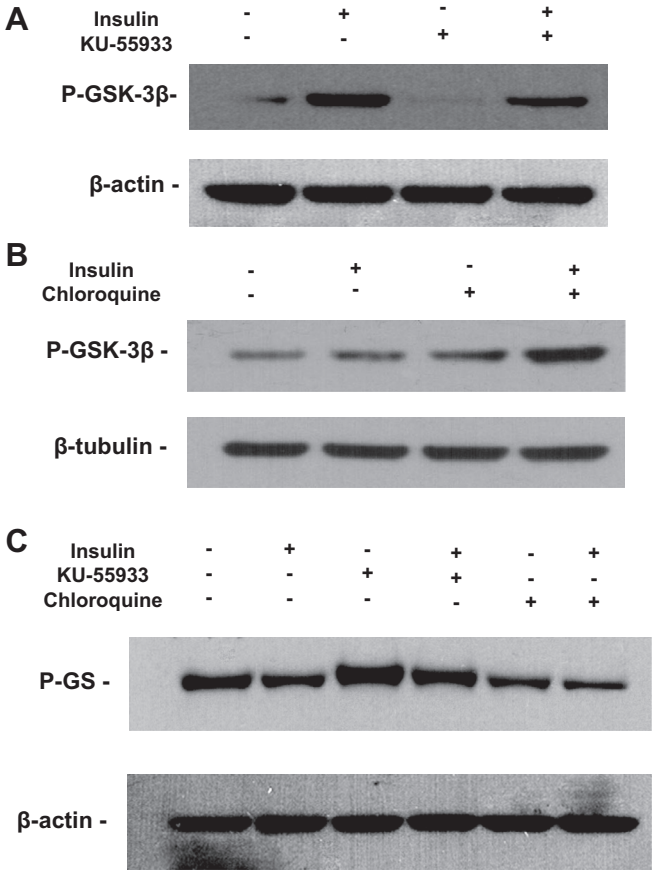

Fig. 3. A. Comparison of GSK-3β phosphorylation in L6 cells treated with insulin and KU-55933. L6 myoblasts were serum-starved overnight, and then treated with KU-55933 (10 μM) for 1 h before the addition of 100 nM insulin for another 45 min. Cells were then lysed and protein samples were subjected to SDS–PAGE and Western blotting. Membranes were incubated with antibodies against phospho-GSK-3β(Ser9) and β-actin, respectively. B. Comparison of GSK-3β phosphorylation in L6 cells treated with insulin and chloroquine. L6 myoblasts were serum-starved overnight, and then treated with chloroquine and insulin as described in Fig. 2B. Cells were then lysed and protein samples were subjected to SDS–PAGE and Western blotting. Membranes were incubated with antibodies against phospho-GSK-3β(Ser9) and β-tubulin, respectively. C. Comparison of GS phosphorylation in L6 cells treated with chloroquine and KU-55933. L6 myoblasts were serum-starved overnight, and then treated with chloroquine, KU-55933, and insulin as described above. Cells were then lysed and protein samples were subjected to SDS–PAGE and Western blotting. Membranes were incubated with antibodies against phospho-GS(Ser641) and β-actin, respectively. The results in A-C are representative of 3 separate experiments.

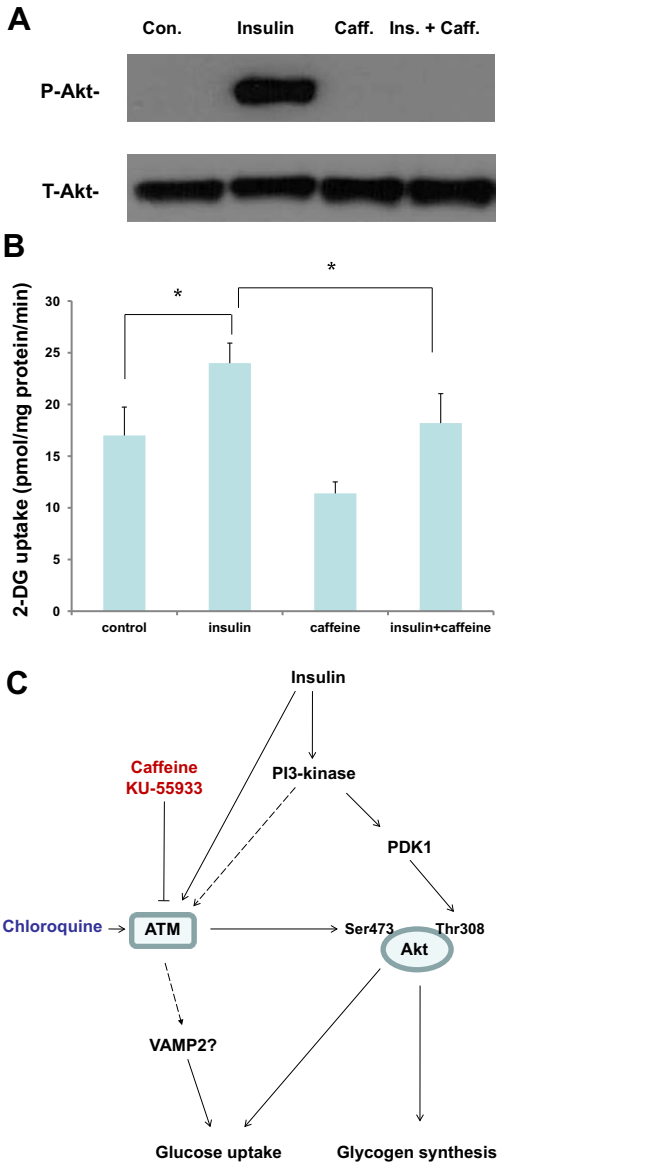

Fig. 4. A. Caffeine inhibits Akt phosphorylation following insulin treatment in L6 cells. Subconfluent L6 myoblasts were serum-starved overnight and then treated with caffeine (300 μM) for 1 h before the addition of 100 nM insulin for 45 min. Cells were then lysed and samples were subjected to SDS–PAGE. Levels of phospho-Akt (Ser473) and total Akt were determined by immunoblotting. The results presented are representative of 3 separate experiments. B. Caffeine inhibits insulin-mediated glucose uptake in L6 cells. Subconfluent L6 myoblasts were serum-starved for 6 h, then incubated with or without caffeine followed by insulin treatment as described above. Following treatment, a 2-DG incorporation assay was performed as described in Materials and Methods. The bar graph indicates the averages ± SEM of the 2-DG uptake values from 3 separate experiments (*$p < 0.05$). C. Potential roles of different modulators of ATM in Akt phosphorylation, glucose uptake, and glycogen synthesis in muscle cells. This figure summarizes the current knowledge/hypotheses about the effect of chloroquine and other modulators of ATM on Akt and glucose homeostasis in muscle cells. Dashed lines represent our hypotheses that require further testing.

stimulate glycogen synthesis by incorporating glucose into glycogen. Since ATM affects phosphorylation of GSK-3, we examined the effects of KU-55933 and chloroquine on phosphorylation of GS. As shown in Fig. 3C, under the basal condition, GS showed a strong phosphorylation signal, whereas the addition of insulin led to a reduction in GS phosphorylation. However, KU-55933 treatment resulted in a higher level of GS phosphorylation, even in the presence of insulin. By contrast, the addition of chloroquine resulted in decreased phosphorylation of GS, indicating elevated glycogen synthase activity and enhanced glycogen synthesis.

3.6. Caffeine inhibits Akt phosphorylation and glucose uptake following insulin treatment in L6 cells

To further confirm chloroquine's hypoglycemic effect through ATM, other modulators of ATM activity, such as caffeine, was also tested in L6 muscle cells. Recent reports found that caffeine intake in human leads to an increase in blood glucose levels and a decrease in insulin sensitivity [20]. Further studies showed that caffeine results in decreased glucose disposal in both skeletal muscle and adipose tissue of human subjects [21]. However, the mechanism underlying caffeine's effect on glucose homeostasis is still unclear. In light of caffeine's role as a strong inhibitor of ATM [22], we tested its effect on Akt phosphorylation and glucose uptake in L6 cells. Caffeine completely abrogated phosphorylation

of Akt at Ser473 (Fig. 4A). Likewise, we also found that treatment with caffeine led to a decrease in 2-deoxyglucose uptake in L6 muscle cells (Fig. 4B). Therefore, these results suggest that the effect of caffeine on glucose homeostasis in the human body may result from its inhibitory effect on ATM kinase activity and Akt phosphorylation, thereby suppressing insulin-mediated glucose uptake.

4. Discussion

In addition to its hypoglycemic effect in animal models of T2D [1,2], chloroquine was also tested in multiple human pilot clinical trials for T2D patients. These trials have reported significant improvements in insulin sensitivity and glucose tolerance in patients treated with chloroquine [23,24]. However, the clinical usage of chloroquine for patients with T2D has been hindered by the lack of a clear mechanism underlying its glucose regulatory function. In this report, our results provide strong evidence showing that chloroquine achieves these effects through activation of Akt and its downstream targets.

Our results demonstrate, for the first time, that chloroquine is an activator of Akt *in vitro* in cultured L6 muscle cells and *in vivo* in a rat model of insulin resistance. In addition to demonstrating that chloroquine is a novel activator of Akt, our results also show that it can trigger signaling events downstream of Akt. One such event stimulated by chloroquine is the insulin-mediated glucose uptake in muscle cells.

Moreover, while chloroquine did not result in a detectable increase in basal Akt phosphorylation at Ser473 in L6 cells, it did result in a dramatic increase in glucose uptake even without the addition of insulin. These results suggest an Akt-independent effect of ATM on glucose uptake. In fact, this observation is supported by several lines of evidence. First, it was discovered that a fraction of cytoplasmic ATM associates with cytoplasmic vesicles and binds to the components, such as β-adaptin, within the vesicles [9]. Second, a large scale proteomic analysis of potential substrates of ATM found that VAMP2, a key protein directly involved in the translocation of glucose transporter 4 (GLUT4), is a potential substrate of ATM [14].

GLUT4 is a major regulator of glucose uptake in muscle cells. It functions by translocating glucose from the cell membrane into the cytosol [16]. It is thus possible that ATM, in addition to activating Akt, can also directly bind to GLUT4-storage vesicles (GSVs) and phosphorylate VAMP2 within the GSVs. The activation of VAMP2 can then lead to translocation of GLUT4 to the cell membrane [16]. Thus, chloroquine treatment alone can mimic the effects of insulin. Meanwhile, treatment with both insulin and chloroquine led to a stronger increase of glucose uptake, suggesting that chloroquine has the potential to result in maximal stimulation of glucose uptake when combined with insulin.

In muscle cells, glucose utilization is not only regulated by glucose uptake, but also by glycogen synthesis. Indeed, impaired glycogen synthesis is a major contributor to the pathophysiology of T2D [18]. Studies have found that inhibition of GSK-3 (by an inhibitor) or deficiency of GSK-3 (by gene knockout) is sufficient to activate GS and improve insulin sensitivity [25,26]. Because inhibition of GSK-3, though not affecting glucose uptake, can independently lead to improved insulin sensitivity, development of novel GSK-3 inhibitors is also considered a promising therapeutic strategy for the treatment of T2D. Thus, besides stimulating glucose uptake, chloroquine's ability to inhibit GSK-3 makes it a more attractive and potentially more effective therapeutic agent for the treatment of T2D (Fig. 4C).

Chloroquine has proven to be safe and successful for the treatment of malaria in humans. Our studies also demonstrate that chloroquine, through its activation of Akt, strongly promotes glucose uptake and glycogen synthase activity in muscle cells. Therefore, our findings may facilitate clinical investigations of chloroquine as a highly effective therapeutic agent for the treatment of insulin resistance and T2D. Furthermore, chloroquine is known to reduce the risk of developing cancer and cardiovascular diseases. For instance, patients who are on chloroquine for the treatment of malaria also have a significant reduction in the incidence of endemic Burkitt lymphoma [27]. Moreover, while A-T patients and heterozygosity of ATM are associated with elevated risk of cardiovascular diseases, $ATM^{+/+}$ and $apoE^{-/-}$ mice treated with choloroquine exhibit decreases in western-diet induced atherosclerosis [4]. Long term use of growth hormones, such as insulin and GLP-1, as treatment regimens for T2D is associated with increased risks of cancer and cardiovascular diseases [28]. Therefore, chloroquine, either used alone or in combination with insulin or GLP-1 may have additional benefits for patients with T2D and other associated risk factors.

The cytoplasmic function of ATM in insulin signaling and regulation of glucose homeostasis is now being well recognized [5,6,15]. While ATM's role in activation of Akt makes it an attractive new therapeutic target for T2D [15], the results presented in this report regarding chloroquine's stimulatory effects on glucose uptake and glycogen synthesis have further validated ATM as a promising target. There is no doubt that these findings, along with other known beneficial effects of chloroquine, may trigger the screen for other novel activators of ATM, leading to promising new treatment regimes for T2D and other human diseases.

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