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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

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