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Original Paper

Chloroquine Increases Glucose Uptake via Enhancing GLUT4 Translocation and Fusion with the Plasma Membrane in L6 Cells

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Key Words
Chloroquine • Glucose uptake • GLUT4 • Ca^{2+}

Abstract
Background/Aims: Chloroquine can induce an increase in the cellular uptake of glucose; however, the underlying mechanism is unclear. ***Methods:*** In this study, translocation of GLUT4 and intracellular Ca^{2+} changes were simultaneously observed by confocal microscope in L6 cells stably over-expressing IRAP-mOrange. The GLUT4 fusion with the plasma membrane (PM) was traced using HA-GLUT4-GFP. Glucose uptake was measured using a cell-based glucose uptake assay. GLUT4 protein was detected by Western blotting and mRNA level was detected by RT-PCR. ***Results:*** We found that chloroquine induced significant increases in glucose uptake, glucose transporter GLUT4 translocation to the plasma membrane (GTPM), GLUT4 fusion with the PM, and intracellular Ca^{2+} in L6 muscle cells. Chloroquine-induced increases of GTPM and intracellular Ca^{2+} were inhibited by Gallein ($G_{\beta\gamma}$ inhibitor) and U73122 (PLC inhibitor). However, 2-APB (IP_3R blocker) only blocked the increase in intracellular Ca^{2+} but did not inhibit GTPM increase. These results indicate that chloroquine, via the $G_{\beta\gamma}$-PLC-IP_3-IP_3R pathway, induces elevation of Ca^{2+}, and this Ca^{2+} increase does not play a role in chloroquine-evoked GTPM increase. However, GLUT4 fusion with the PM and glucose uptake were significantly inhibited with BAPTA-AM. This suggests that Ca^{2+} enhances GLUT4 fusion with the PM resulting in glucose uptake increase. ***Conclusion:*** Our data indicate that chloroquine via $G_{\beta\gamma}$-PLC-IP_3-IP_3R induces Ca^{2+} elevation, which in turn promotes GLUT4 fusion with the PM. Moreover, chloroquine can enhance GLUT4 trafficking to the PM. These mechanisms eventually result in glucose uptake increase in control and insulin-resistant L6 cells. These findings suggest that chloroquine might be a potential drug for improving insulin tolerance in diabetic patients.

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Introduction

There are more than 340 million people with type 2 diabetes mellitus (T2DM) in the world [1]. We need to find effective antidiabetic agents to treat T2DM. Chloroquine is a synthetic bitter-tasting compound that has been used to treat patients with malaria [2] and to inhibit autophagy to protect vascular function during nutrient deprivation [3]. In addition, this bitter tastant can also improve glucose metabolism in patients with insulin-resistant diabetes mellitus [4] and significantly increase glucose uptake mediated by Akt in L6 muscle cells [4, 5]. Therefore, chloroquine is a potential therapeutic agent for patients with type 2 diabetes mellitus; however, the underlying mechanism needs to be further defined.

Glucose uptake is mainly dependent on glucose transporter 4 (GLUT4) which translocates extracelluar glucose through the cell membrane into the cell. GLUT4 is important for preserving whole-body glucose homeostasis [6]. Selective disruption of GLUT4 expression in muscle or adipose tissue induces global insulin resistance [7-10]. GLUT4 heterozygous Knockout mice developed muscle insulin resistance and diabetes [11], whereas transgenic over-expression of GLUT4 improved glucose utilization rate *in vivo* [9]. Therefore, GLUT4 is considered to be a glucose sensor and an important therapeutic target for the treatment of type 2 diabetes mellitus.

GLUT4 is mainly found in intracellular GLUT4 storage vesicles (GSVs). Insulin induces a rapid translocation of GSVs from the trans-Golgi network (TGN) and/or endosomes to the plasma membrane (PM) and then fusion with the PM, resulting in an increase of glucose uptake [12]. This process is up-regulated by insulin receptor/insulin receptor substrate-1 (IRS-1), phosphatidylinositol 3 kinase (PI3-K), protein kinase B (PKB/Akt), atypical protein kinase C (aPKC) [13], and cytosolic Ca^{2+} [14]. However, it is unclear whether and how chloroquine increases glucose uptake via the GLUT4 pathway.

In the present study, we found that chloroquine increases glucose uptake through enhancing GSVs fusion with the cell membrane by activating the G protein-PLC-IP_3R-Ca^{2+} pathway. This study also provides an efficient cell-based method for identifying potential antidiabetic medicines from synthetic and natural bitter tastants.

Materials and Methods

Reagents

GLUT4 antibody was purchased from Cell Signaling Technology (Beverly, MA, USA). HA antibody was purchased from TransGen Biotech (Beijing, China). Fluo-4 AM was purchased from Invitrogen (Camarillo, CA, USA). 3-[1-[3-(Dimethylamino) propyl]-5-methoxy-1H-indol-3-yl]-4-(1H-indol-3-yl)-1H-pyrrole-2,5-dione (Gö6983) was purchased from EMD Millipore (Billerica, MA). Gallein was purchased from Tocris Bioscience (Bristol, UK). 1,2-bis (*o*-aminophenoxy) ethane-*N*,*N*,*N*',*N*'-tetraacetic acid tetra (acetoxymethyl) ester (BAPTA-AM), 1-(6-((8R,9S,13S,14S,17S)-3-methoxy-13-methyl-7, 8, 9, 11,12,13,14,15,16,17-decahdro-6H-cyclopenta[a]phenanthren-17-ylamino)hexyl)-1H-pyrrole-2,5-dione (U73122), 2-Aminoethoxydiphenyl borate (2-APB), chelerythrine chloride (Che) and palmitate were purchased from Sigma (St. Louis, MO, USA). Fluo-4 AM, Gö6983, Gallein, BAPTA-AM, U73122, 2-APB, Che were dissolved in dimethylsulphoxide (DMSO) and then diluted in physiological saline solution (PSS).

Solutions

The PSS contained (mM): 135 NaCl, 5 KCl, 1 $MgCl_2$, 2 $CaCl_2$, 10 HEPES, and 10 glucose (pH = 7.4 adjusted with NaOH). Ca^{2+}-free PSS contained (mM): 135 NaCl, 5 KCl, 1 $MgCl_2$, 0.5 EGTA, 10 HEPES, and 10 glucose (pH = 7.4).

Plasmid and cell line construction

The Aminopeptidase IRAP has been identified as a major protein that co-localizes with GLUT4 in insulin-responsive GSVs [15, 16] and has been successfully used as a reporter molecule to analyze insulin-regulated GLUT4 trafficking [17, 18]. In this study, IRAP and mOrange were inserted into the

pQCXIP plasmid. The retrovirus was prepared by transfecting pQCXIP-IRAP-mOrange, vesicular stomatitis virus G (VSVG), and PHIT60 (include MuLV structural genes, namely gag and pol) at a ratio of 2:1:1 using Lipofectamine 2000 (Invitrogen, CA, USA) into Plat E cells [19]. The cultural supernatant was collected after 48 h. The viruses were concentrated by super-centrifugation (at 50,000 g, 30 min). L6 cells (presented by Professor Pingsheng Liu, Institute of Biophysics, the Chinese Academy of Science) were infected with freshly prepared viruses during the exponential growth phase. Polybrene (8 μg/ml) was used to facilitate efficient viral infection. Cells with fluorescence were isolated by fluorescence activated cell sorter (FACS) and a single cell per well was seeded into 96-well plates. Finally, a single clone with the highest fluorescence intensity was selected, following stimulation with insulin (100 nM).

Cell culture and development of insulin-resistant cells

L6 cells were cultured in minimum essential medium alpha modification (MEM-α, Gibco, USA) supplemented with 10 % fetal bovine serum (FBS, Hyclone, USA) and 1% antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin) at 37°C in 5 % CO_2. For differentiation into myotubes, cells were cultured in MEM-α supported with 2 % FBS and the medium replaced every 48 h. On day 7, L6 cells were used for experiments. High concentration insulin or palmitate was used to obtain insulin resistance in L6 myotubes. To obtain insulin resistance by high concentration insulin, cells were cultured with the addition of 1 μM insulin for 24 h and control cells were incubated with normal α-MEM medium. To obtain insulin resistance by high concentrations of palmitate, a stock concentration of palmitate was dissolved in 50% ethanol and conjugated with 10% FFA-free BSA. Insulin resistance in L6 myotubes was then induced by treatment with BSA conjugated palmitate (0.5 mM) for 16 h. Control cells were treated with vehicle containing BSA but lacking the fatty acid [20].

Assays of GLUT4 translocation

L6 cells stably expressing IRAP-mOrange (L6 IRAP-mOrange) were cultured in α-MEM supplemented with 10% fetal bovine serum at 37°C in 5% CO_2. Before strating the experiment, L6 IRAP-mOrange was seeded onto glass coverslips for overnight, and then starved in serum-free α-MEM for 2 h. The cells were imaged with a laser-scanning confocal microscope LSM 700 (Carl Zeiss, Jena, Germany) to monitor the dynamics of IRAP-mOrange translocation. Images were taken after addition of 100 nM insulin or 100 μM chloroquine, using 555 nm excitation laser every 5 min in 30 min. The fluorescence intensity of IRAP-mOrange at the PM was quantified as previously described [21] and was used to reflect the GLUT4 translocation [16].

Measurements of intracellular Ca^{2+}

Intracellular Ca^{2+} was similarly measured and analyzed as previously described [22], with some modifications. L6 cells were incubated in PSS containing 2 μM fluo-4 AM for 15 min at room temperature in the microscope recording chamber and then were superfused with dye-free PSS for 10 min. The excitation wavelength was provided at 488 nm and the emitted fluorescence of fluo-4 AM was imaged through a 505 nm filter. The fluorescence intensity of fluo-4 AM was measured by a LSM 700 laser scanning confocal system (Carl Zeiss, Jena, Germany). Images were taken every 5 min in 30 min after addition of 100 μM chloroquine. Fluorescence intensity of intracellular Ca^{2+} concentration was analyzed using the Zen 2010 software (Carl Zeiss, Jena, Germany).

Assay of GLUT4 fusion with the PM

pQBI25-HA-GLUT4-GFP [21] were transfected into L6 cells using Lipofectamine (Invitrogen, CA, USA). L6 cells were incubated in the absence or presence of chloroquine for 30 min, which were then fixed with 3% paraformaldehyde, blocked in 2% (w/v) BSA in PBS, and incubated with mouse anti-HA antibody (1:200 dilution). Cells were subsequently labeled with anti-mouse-PE-conjugated secondary antibody [23]. The intensity of Green fluorescent protein (GFP) and P-phycoerythrin (PE) fluorescence was measured by confocal microscope and images were processed using the Zen 2010 software.

Western blotting analysis

Differentiated L6 cells were prepared and harvested following starvation for 2 h. The cells were then washed with 5 ml of cold PBS, resuspended in HES buffer (mM; 250 Sucrose, 20 HEPES, 10 EDTA, pH 7.4) containing protease inhibitor cocktail (Roche, Basel, CHE) at 4°C. Cells were lysed by passing 12

times through a 22-gauge needle followed by 6 additional passes through a 27-gauge needle. The lysate was centrifuged at 500 g for 10 min to remove unbroken cells. All samples were subjected to SDS-PAGE analysis on resolving gels containing 10% acrylamide. Equal amounts of protein were loaded for each sample. Separated proteins were electrophoretically transferred to a Polyvinylidene fluoride (PVDF) membrane, and the membrane was probed with primary and HRP-conjugated secondary antibodies. The protein bands were quantified by ChemiDoc XRS (Bio-Rad, CA, USA).

Glucose uptake assays

Glucose uptake assays were performed on L6 cells using a cell-based glucose uptake assay kit (Cayman Chemical, USA). Cells were seeded into a 96-well plate at a density of $1 \times 10^4 - 5 \times 10^4$ cells/well in 100 µl α-MEM medium. After 12 h incubation, cells were treated with 100 µM chloroquine or 100 nM insulin or vehicle control in 100 µL glucose-free α-MEM medium containing 150 µg/mL 2-NBDG. Plates were incubated at 37°C with 5% CO_2 for 30 min. At the end of the treatment, plates were centrifuged for five minutes at 400 g at room temperature. The supernatant was aspirated and 200 µL of cell-based assay buffer was added to each well. Plates were centrifuged for five minutes at 400 g at room temperature. Then the supernatant was aspirated again and 100 µL of cell-based assay buffer was added to each well. The 2-NBDG taken up by cells was detected using 485nm as excitation wavelength and 535 nm as emission wavelength (Infinite M200 Pro, Tecan, CHE).

RNA isolation and quantitative real-time PCR

The total RNA of differentiated L6 cells was extracted using TRIzol reagent (Invitrogen, CA, USA). The RNA treated with DNase I (Thermo Fisher) to remove genomic DNA. Then cDNA was synthesized from 2 µg RNA using M-MLV Reverse Transcriptase (Promega) in 50 µl. Real-time PCR reactions were performed using the 7500 Fast Real-Time PCR System instrument (Applied Biosystems) and SYBR® Green Realtime PCR Master Mix (TOYOBO) was used in this experiment. The sequences for each primer pairs were as follows: rat Gapdh (NCBI RefSeq NM_017008.4), F: 5'-TACAGCAACAGGGTGGTGGAC-3', R: 5'-GGGATGGAATTGTGAGGGAGA-3'; rat Glut4 (NCBI RefSeq NM_012751.1), F: 5'-CTTCC TTCTATTTGCCGTCCTC-3', R: 5'- GCTGCTGTTTCCTTCATCCTG-3'.

Statistical analysis

All data are presented as the mean ± SEM. The *n* values represent the number of cells. Student's *t*-tests were performed to determine whether there were significant differences between the means using Origin 9.0 software (OriginLab, Northampton, MA, USA). Differences with $p < 0.05$ were considered statistically significant.

Results

Chloroquine increased glucose uptake in L6 cells

In this study, we analyzed the effect of chloroquine on cellular glucose uptake in comparison with insulin-triggered glucose uptake as a positive control. As shown in Fig. 1, following the treatment of L6 cells with 100 nM insulin and 100 µM chloroquine, 2-NBDG uptake increased ~1.8-fold and ~3.0-fold, respectively. Chloroquine treatment in fact

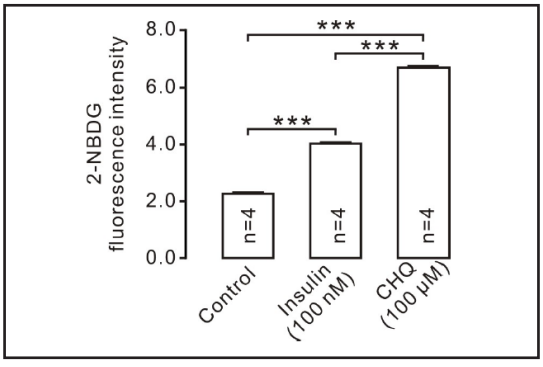

Fig. 1. Chloroquine (CHQ) increased glucose uptake in L6 cells. Glucose uptake was measured by 2-NBDG assay. Insulin and CHQ significantly increased glucose uptake, respectively. The level of CHQ-induced glucose uptake was greater than that induced by insulin. ***: P < 0.001. These results indicate that CHQ can increase glucose uptake.

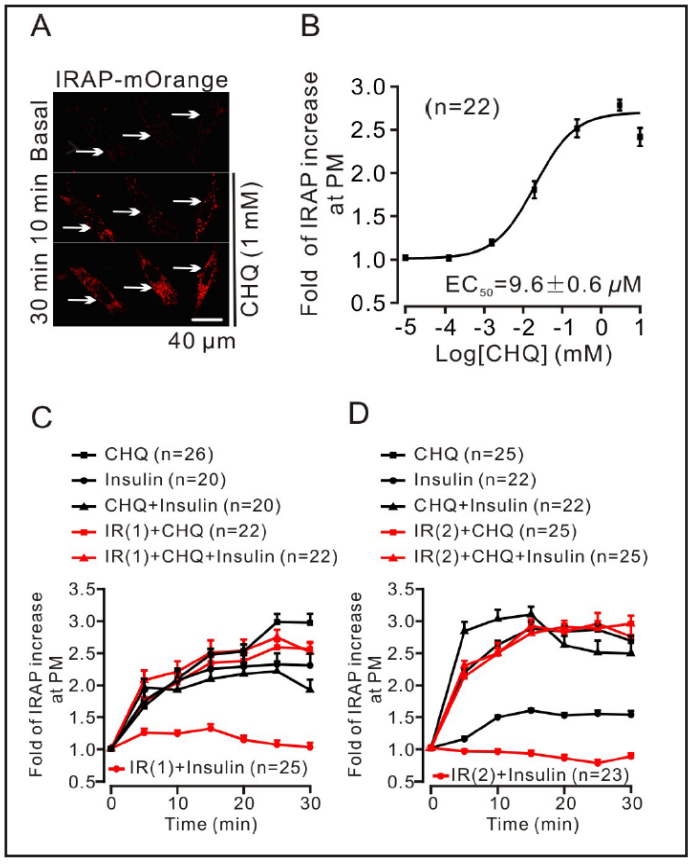

Fig. 2. CHQ enhanced GLUT4 translocation in control and insulin-resistant L6 cells. (A) IRAP fluorescence in L6 cells transfected with pIRAP-mOrange was evaluated by confocal microscope. Following the addition of CHQ, IRAP fluorescence intensity at the PM was increased at 10 and 30 min. Scale bar: 40 μm. (B) Dose-response curve from 22 cells at the 30 min time point. The EC_{50} was 9.6 ± 0.6 μM. (C) Time course of the changes in IRAP fluorescence intensity at the PM following addition of CHQ and insulin in control and insulin-resistant (IR(1)) cells, respectively. Insulin resistance in L6 cells was induced by treatment with 1 μM insulin for 24 h and control cells were incubated with normal α-MEM medium. CHQ reversed high concentration insulin-induced decrease in insulin-stimulated GLUT4 trafficking in L6 cells. (D) Time course of the changes in IRAP fluorescence intensity at the PM following addition of CHQ and insulin in insulin-resistant (IR(2)) and control cells, respectively. Insulin resistance in L6 cells was induced by treatment with 0.5 mM BSA-conjugated palmitate for 16 h and control cells were treated with vehicle containing BSA and ethanol but lacking the fatty acid. CHQ reversed palmitate-induced decrease in insulin-stimulated GLUT4 trafficking in L6 cells. These data demonstrate that CHQ can increase GLUT4 trafficking to the PM in control and insulin-resistant cells.

resulted in a much higher 2-NBDG uptake compared to insulin treatment ($P < 0.01$). Thus, chloroquine strongly enhances glucose uptake.

Chloroquine enhanced GLUT4 trafficking to the PM

To further clarify whether the enhanced glucose uptake was due to chloroquine enhanced GLUT4 trafficking to the PM, we measured the intensity of red fluorescence emitted from mOrange-tagged IRAP (IRAP-mOrange) in L6 cell as a proxy for alterations in GLUT4 presence in the PM. Following the addition of chloroquine, an increase in red fluorescence was observed at the PM (Fig. 2A). This response occurred in a dose-dependent manner in 22 cells (Fig. 2B), which showed that the chloroquine induced increase in IRAP fluorescence and plateaued within 30 min in both insulin-resistant (IR) and non-insulin-resistant (NIR) cells (Fig. 2C and D). High concentration insulin and palmitate were used to obtain insulin resistance in L6 myotubes, respectively. Insulin induced a similar reaction in NIR cells, but not in IR cells. These data imply that chloroquine can enhance GLUT4 trafficking to the PM particularly also in IR cells while insulin has no effect in these cells.

Role of intracellular Ca^{2+} in chloroquine-enhanced GLUT4 trafficking

Previous studies have reported that chelation of intracellular Ca^{2+} using BAPTA-AM resulted in 95% inhibition of insulin-stimulated glucose uptake [24], suggesting that intracellular Ca^{2+} plays a crucial role in insulin-induced glucose uptake. To elucidate whether Ca^{2+} plays a similar role in chloroquine-induced GLUT4 trafficking and glucose uptake, we simultaneously observed the changes in Ca^{2+} and GLUT4 trafficking. Following the addition of

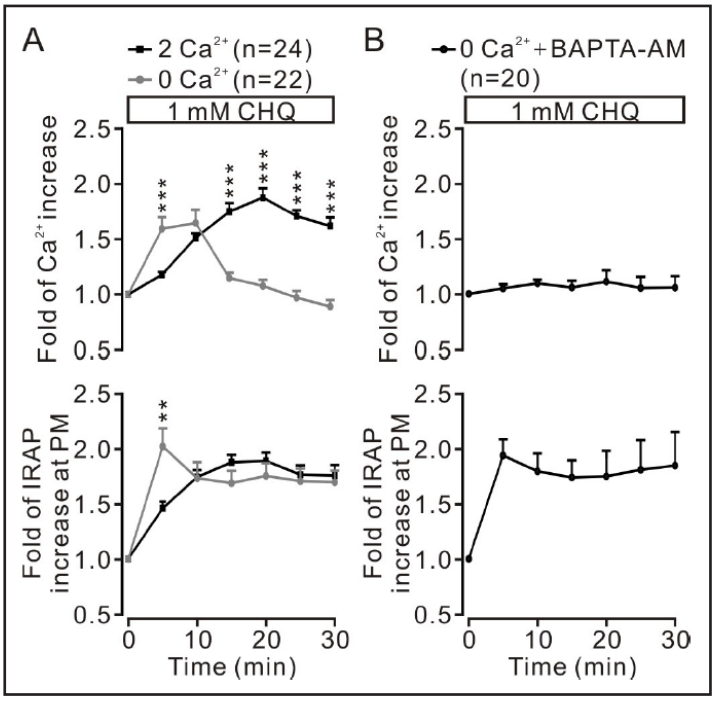

Fig. 3. BAPTA-AM did not inhibit CHQ-stimulated GLUT4 trafficking in L6 cells. (A) Cells were treated with 1 mM CHQ. Changes in intracellular Ca^{2+} (upper) and IRAP fluorescent intensity at the PM (bottom) under 2 mM extracellular Ca^{2+} (n = 24 cells) or 0 mM extracellular Ca^{2+} (n = 22 cells) conditions were shown. (B) The same experiments were performed under 0 mM extracellular Ca^{2+} + 10 μM BAPTA-AM conditions. **: P < 0.01; ***: P < 0.001. These results suggest that CHQ-induced enhancement of GLUT4 trafficking to the PM is not dependent on CHQ-triggered intracellular Ca^{2+} increase.

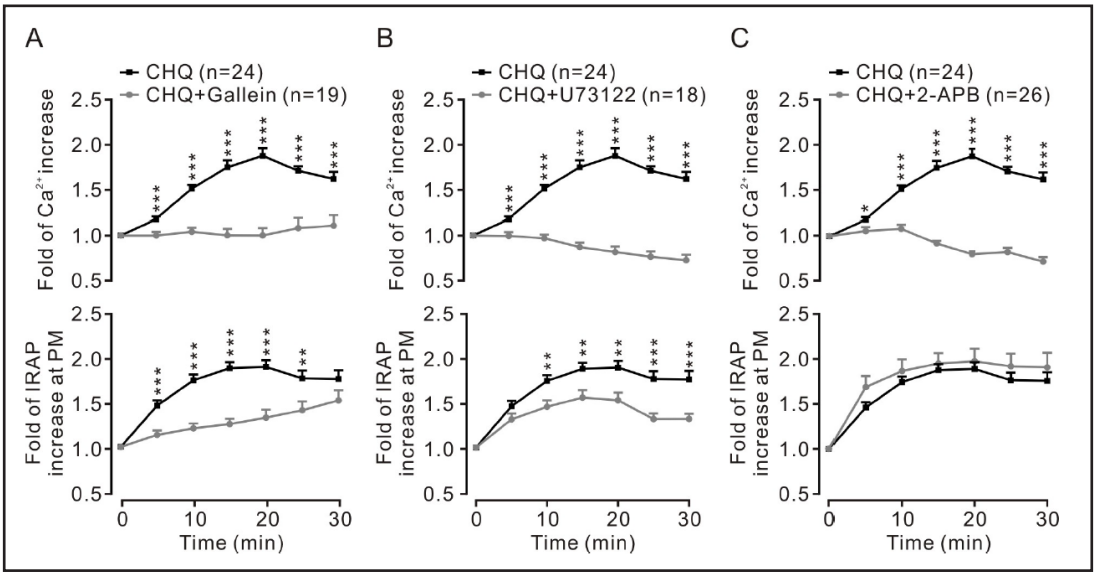

Fig. 4. CHQ, through the G protein-PLC-IP_3 pathway, regulated intracellular Ca^{2+} increase and GLUT4 trafficking. (A) CHQ simultaneously induced IRAP fluorescence increase at the PM and Ca^{2+} elevation in control (n = 24 cells) and Gallein treated L6 cells (100 μM for 6-8 h, n = 19 cells), respectively. (B) Similar experiments were done in control (n = 24 cells) and PLC inhibitor U73122 treated L6 cells (1 μM for 30 min, n = 18 cells). (C) The experiments were performed in control (n = 24 cells) and IP_3 receptor blocker 2-APB incubated cells (100 μM for 30 min, n = 26 cells). These results indicate that CHQ-induced Ca^{2+} increase is not the causal reason for CHQ-evoked GLUT4 trafficking. *: P < 0.05; **: P < 0.01; ***: P < 0.001.

1 mM chloroquine, the level of intracellular Ca^{2+} exhibited either an increase with saturation in the culture medium with 2 mM Ca^{2+} or a transient intracellular Ca^{2+} increase in the culture medium with 0 mM Ca^{2+} and 0.5 mM EGTA (Fig. 3A upper). The IRAP fluorescence intensity at the PM showed a similar saturating increase (Fig. 3A bottom). These data suggest that the increase in IRAP fluorescence could be triggered by an increase in intracellular Ca^{2+}.

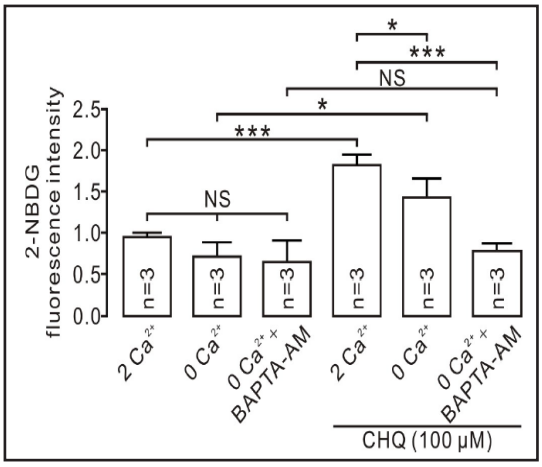

Fig. 5. CHQ increased glucose uptake via Ca^{2+} signaling. Glucose uptake was assayed by measuring the uptake of 2-NBDG. In the absence of CHQ, the glucose uptake was similar under 2 mM extracellular Ca^{2+} or 0 mM extracellular Ca^{2+} + 10 μM BAPTA-AM conditions. In the presence of CHQ, the glucose uptake was significantly increased under 2 mM and 0 mM extracellular Ca^{2+} conditions. However, under 0 mM extracellular Ca^{2+} + 10 μM BAPTA-AM conditions, glucose uptake was inhibited. *: $P < 0.05$; ***: $P < 0.001$. These experiments indicate that CHQ-induced glucose uptake results from CHQ-evoked Ca^{2+} elevation.

To further clarify the role of Ca^{2+} in GLUT4 trafficking, we used BAPTA-AM (10 μM) to chelate Ca^{2+} and thus preventing chloroquine-induced intracellular Ca^{2+} increase (Fig. 3B upper). Under these conditions an increase in IRAP fluorescence could still be observed (Fig. 3B bottom). These results indicate that chloroquine-induced GLUT4 trafficking to the PM may not directly be mediated by an increase in intracellular Ca^{2+}.

Chloroquine via G protein-PLC signaling pathway enhanced GLUT4 trafficking

We next investigated the mechanism underlying chloroquine-induced GLUT4 trafficking. It has been reported that the bitter tastant chloroquine, can activate the bitter taste receptor (T2R) and its coupled downstream signaling pathway via G protein-PLC-IP_3-IP_3R, results in an induction of Ca^{2+} release from the sarcoplasmic reticulum (SR) [25, 26]. Therefore, we studied the effect of this signaling pathway on chloroquine-induced alterations in intracellular Ca^{2+} and chloroquine-induced GLUT4 trafficking to the PM. Treatment of cells with $G_{\beta\gamma}$ protein inhibitor Gallein (100 μM) for 6-8 h or PLC inhibitor U73122 (1 μM) for 30 min completely inhibited the chloroquine-induced rise in cytosolic Ca^{2+} (Fig. 4A and B upper). Also, the IRAP fluorescence intensity at the PM was partially reduced compared to the control (Fig. 4A and B bottom). However, when we used the IP_3R blocker 2-APB (100 μM) to block the chloroquine-induced rise in cytosolic Ca^{2+} (Fig. 4C upper), the increase of IRAP fluorescence intensity at the PM was not affected (Fig. 4C bottom). These data indicate that the G protein-PLC-IP_3-IP_3R pathway somehow mediates the chloroquine-induced rise in cytosolic Ca^{2+}. However this increase in cytosolic Ca^{2+} will not enhance G protein-PLC pathway-mediated GLUT4 translocation to the PM.

Chloroquine-induced Ca^{2+} increase enhanced glucose uptake

Our data showed that the chloroquine-induced rise in cytosolic Ca^{2+} did not promote GLUT4 trafficking to the PM, but chloroquine did enhance the uptake of glucose. Therefore, we next investigated whether Ca^{2+} is required for glucose uptake. Thus, we examined chloroquine-induced glucose uptake under various conditions (Fig. 5). In the absence of chloroquine, there was no significant difference in glucose uptake under conditions with 2 mM extracellular Ca^{2+} or 0 mM extracellular Ca^{2+} in the culture medium. However, upon the addition of chloroquine glucose uptake significant increased regardless of extracellular Ca^{2+} concentration. On the contrary, under conditions with 0 mM Ca^{2+} and 10 μM BAPTA-AM, chloroquine-induced glucose uptake was significantly inhibited. This indicates that Ca^{2+} is essential for chloroquine-induced glucose uptake, however, Ca^{2+} seems not to be required for chloroquine-induced GLUT4 trafficking to the PM.

BAPTA-AM inhibited chloroquine-induced fusion of GLUT4 at the PM

To further understand the mechanism of Ca^{2+}-induced glucose uptake, we studied the effect of Ca^{2+} on GLUT4 insertion into the PM. In the absence of insulin or chloroquine, there

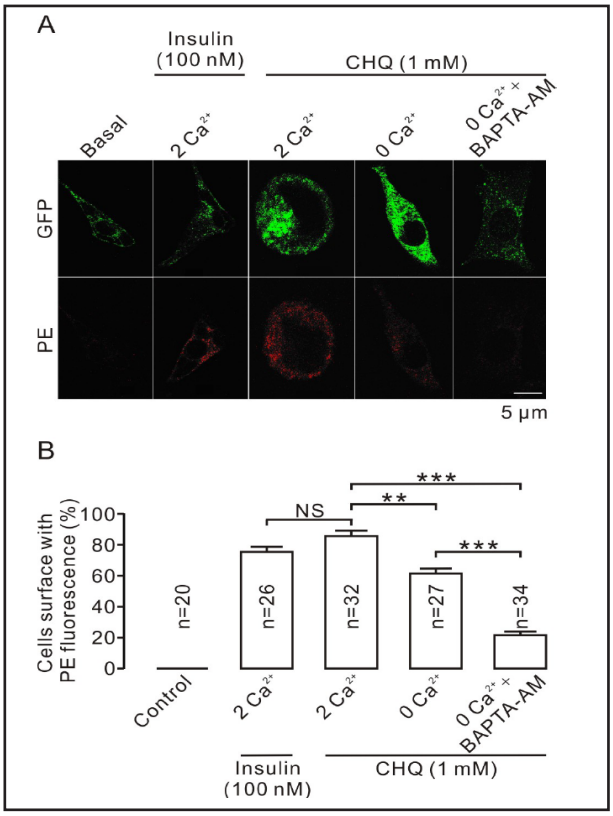

Fig. 6. CHQ increased GLUT4 fusion with the PM via Ca^{2+} signaling. (A) L6 cells were transfected with plasmid pQBI25-HA-GLUT4-GFP encoding a GFP fusion protein with HA epitope-tagged GLUT4 (HA-GLUT4-GFP). Cells were stimulated with the indicated conditions, then fixed and subjected to indirect immunofluorescence antibody staining. PE fluorescence was measured. Scale bar: 5 μm. (B) Among GFP positive population, the percentage of PE positive cells was quantitated. **: $P < 0.01$; ***: $P < 0.001$. These results indicate that CHQ-induced enhancement of GLUT4 fusion with the PM is dependent on intracellular Ca^{2+}.

was no detectable labeling of the anti-HA antibody on the cell surface in cells expressing HA-GLUT4-GFP (Fig. 6A and B). However, in insulin (100 nM) or chloroquine (1 mM) treated cells, we observed a significant increase of PE fluorescence on the cell surface (Fig. 6A and B). Under conditions with 0 mM extracellular Ca^{2+} and 10 μM BAPTA-AM, the chloroquine-induced PE fluorescence increase on the cell surface was completely inhibited. This indicates that Ca^{2+} could be important for the fusion of GLUT4 vesicles to the PM, but not essential for GLUT4 trafficking to the PM.

Chloroquine increased GLUT4 protein and mRNA level

The intensity of IRAP fluorescence in the PM increased following addition of chloroquiue, implying that the amount of GLUT4 also increased. Hence, we measured GLUT4 protein and mRNA level and found both of them to be significantly increased in the presence of insulin or chloroquine (Fig. 7A and B).

Discussion

In the present study, our data suggest that chloroquine through the G protein-PLC-IP_3-IP_3R-Ca^{2+} pathway, promotes GLUT4 vesicles fusion with the PM, and via an unknown pathway enhances GLUT4 trafficking to the PM, thereby resulting in enhanced glucose uptake in L6 cells.

It has been reported that chloroquine activates of the bitter taste receptor (TAS2R) resulting in an increase of intracellular Ca^{2+} through the $G_{\beta\gamma}$-PLC_{β}-IP_3-IP_3R signaling pathway in airway smooth muscle (ASM) [25, 26]. Therefore, we investigated whether and how this signaling pathway mediates an increase in chloroquine-induced glucose uptake (Fig. 1). Glucose uptake rate can be determined by GLUT4 trafficking and inserting into the PM. Our data show that $G_{\beta\gamma}$ inhibitor, Gallein, completely blocked chloroquine-induced intracellular Ca^{2+} increase, but failed to totally inhibit GLUT4 trafficking to the PM (Fig. 4A). This indicates

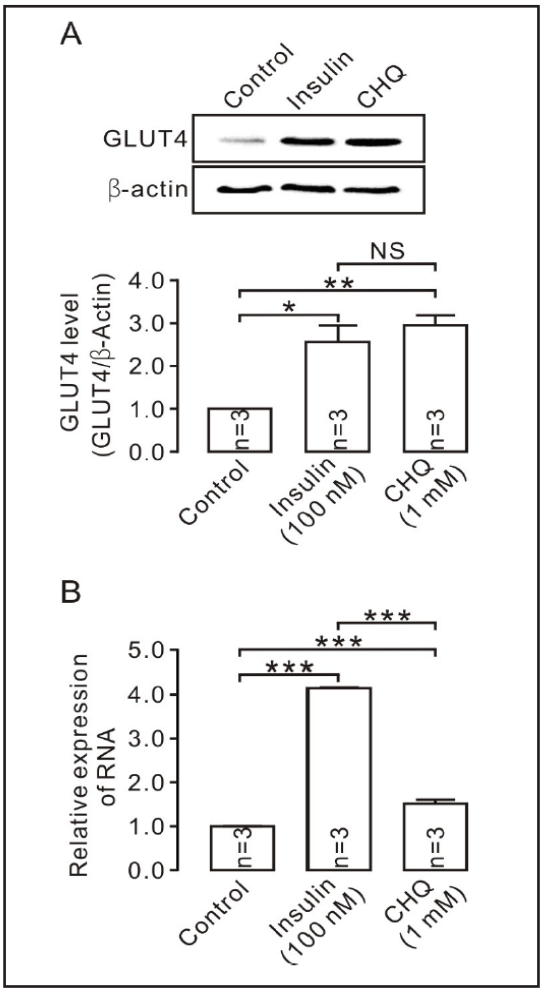

Fig. 7. CHQ induced an increase in GLUT4 protein level. (A) Insulin or CHQ induced an increase in the level of GLUT4 proteins compared to β-actin. Summary results from 3 experiments. *: P < 0.05; **: P < 0.01. These data indicate that CHQ can result in GLUT4 expression increase. (B) Insulin or CHQ induced an increase in the level of mRNA compared to control. ***: P < 0.001. These results were from 3 independent experiments.

that chloroquine can induce intracellular Ca^{2+} increase via the $G_{\beta\gamma}$-mediated signaling pathway as previously reported [25, 26], and that the increase in Ca^{2+} likely mediates chloroquine-induced GLUT4 trafficking to the PM. This conclusion was further supported by the experiments performed on PLC inhibitor U73122-treated cells (Fig. 4B). However, when utilizing 2-APB to inhibit IP_3R, a downstream of PLC, we observed a different phenomenon. 2-APB failed to inhibit GLUT4 trafficking, even though 2-APB blocked chloroquine-induced intracellular Ca^{2+} increase (Fig. 4C). These results indicate that chloroquine-caused intracellular Ca^{2+} elevation does not play a role in chloroquine-evoked GLUT4 trafficking to the PM. In other words, the partial inhibition of GLUT4 trafficking to the PM by Gallein (Fig. 4A) and U73122 (Fig. 4B) may not due to inhibition of Ca^{2+} elevations induced by chloroquine. This interpretation is also supported by results shown in Fig. 3, in which chloroquine-induced enhancement of GLUT4 trafficking to the PM was not affected by blocking chloroquine-triggered intracellular Ca^{2+} increases. Based on previous results [5, 27], it is likely that chloroquine-induced enhancement of GLUT4 trafficking to the PM is regulated by the Akt pathway. Consistent with our results, chloroquine-induced Ca^{2+} elevations were mediated by the G protein- PLC-IP_3-IP_3 receptor-Ca^{2+} release pathway in the tongue and airway smooth muscle (ASM) cells [26, 28]. However, in murine $CD4^+$ thymocytes, chloroquine blocked the IP_3-induced intracellular Ca^{2+} increase [29]. Such discrepancy might be due to the tissue difference. Moreover, IP_3Rs have three isoforms (IP_3R1, IP_3R2, and IP_3R3) in mammals [30, 31] and IP_3R3 is the main receptor for mediating bitter tastant to induce Ca^{2+} rise in taste cells [32]. Therefore, IP_3R3 might play a similar role in chloroquine-triggered Ca^{2+} increases in L6 cells.

Moreover, the elevation in intracellular Ca^{2+} induced by chloroquine enhances GLUT4 vesicles fusion with the PM and glucose uptake. This is shown in Fig. 5 and 6, where intracellular Ca^{2+} elevations were abolished by the conditions of 0 mM extracellular Ca^{2+} and 10 μM BAPTA-AM (where intracellular Ca^{2+} elevations were completely inhibited as shown in Fig. 3B upper). These results indicated that Ca^{2+} will be required for chloroquine-induced GLUT4 vesicles fusion with the PM and glucose uptake increases in L6 cells. The similar studies were also reported in 3T3-L1 adipocytes [24] and L6 muscle cells [14]. In L6 muscle cells, the Ca^{2+} ionophore ionomycin raised cytosolic Ca^{2+} and caused a gain of GLUT4 in cell surface, and which were reversed by Ca^{2+} chelators EGTA and BAPTA-AM) [14]. In addition, chloroquine induced GLUT4 expression and transcription increase (Fig. 7), which will provide more GLUT4 to traffick to the PM, resulting in glucose uptake increase.

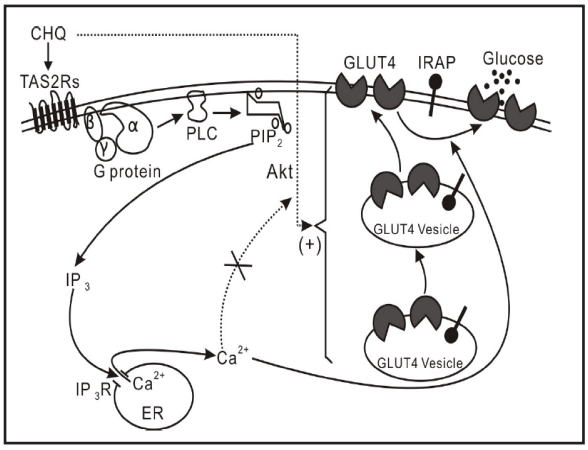

Fig. 8. A model is proposed of the CHQ-induced increase of glucose uptake. CHQ via the TAS2R-$G_{\beta\gamma}$-PLC-IP_3-IP_3R-Ca^{2+} pathway enhances GLUT4 vesicles fusion with the PM and via Akt pathway increases GLUT4 translocation to the PM, eventually resulting in an increase of glucose uptake.

In summary, while previous results suggest that chloroquine-induced enhancement of GLUT4 trafficking to the PM is through the Akt pathway [5, 27], our data have further demonstrated that chloroquine increases glucose uptake by enhancing GLUT4 fusing with the PM through the TAS2R-$G_{\beta\gamma}$-PLC-IP_3-IP_3R-Ca^{2+} pathway (Fig. 8).

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Disclosure Statement

The authors declare no conflict of interests.

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