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Pyrroloquinoline quinone, a novel protein tyrosine phosphatase 1B inhibitor, activates insulin signaling in C2C12 myotubes and improves impaired glucose tolerance in diabetic KK-A^y mice

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ABSTRACT

Insulin resistance is a pathological hallmark of type 2 diabetes mellitus and is characterized by defects in insulin signaling. Protein tyrosine phosphatase 1B (PTP1B) negatively regulates insulin signaling by tyrosine dephosphorylation of insulin receptor, and increased activity and expression of PTP1B is implicated in the pathogenesis of insulin resistance. Therefore, inhibition of PTP1B is anticipated to improve insulin resistance in type 2 diabetic subjects. Pyrroloquinoline quinone (PQQ), a redox cofactor for bacterial dehydrogenases, inhibits PTP1B to oxidatively modify the catalytic cysteine through its redox cycling activity. Here, we report that PQQ induces the ligand-independent activation of insulin signaling by inhibiting cellular PTP1B and enhances glucose uptake through the translocation of glucose transporter 4 in mouse C2C12 myotubes. Furthermore, we demonstrated that oral administration of PQQ improved impaired glucose tolerance in type 2 diabetic KK-A^y mice. Our results strongly suggest that PQQ can be useful in anti-diabetic treatment for type 2 diabetic subjects.

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1. Introduction

The prevalence of type 2 diabetes mellitus (T2DM) has increased dramatically in recent decades worldwide, and the morbidity and mortality associated with secondary complications of the disease such as retinopathy, nephropathy, and cardiovascular disease have also elevated significantly [1]. T2DM is a common metabolic disorder characterized by chronic hyperglycemia and dyslipidemia resulting from insulin resistance of the peripheral tissues and impaired insulin secretion from the pancreas [2]. Glycemic control is the basis for the treatment of T2DM, as it can prevent or slow the progress of associated complications [3]. Relieving insulin resistance has been considered as a primary strategy to improve metabolic control in T2DM subjects [4]. The molecular mechanism underlying insulin resistance is highly complicated but there is evidence of a defect in insulin signaling [5].

When insulin binds to insulin receptor (IR), the intrinsic tyrosine kinase activity becomes activated and autophosphorylates several tyrosine residues. Then, the activated IR phosphorylates IR substrate-1 (IRS-1) at key tyrosine residues and in turn triggers signaling transduction by activating downstream targets such as phosphatidylinositol-3 kinase (PI3K) and Akt. The activated Akt induces the translocation of glucose transporter 4 (GLUT4) from the intracellular compartment vesicle to the plasma membrane, leading to increased cellular glucose uptake. On the other hand, protein tyrosine phosphatase 1B (PTP1B) is known to be a key negative regulator of insulin signaling [6]. This phosphatase catalyzes dephosphorylation of tyrosine residues in IRβ subunit and IRS-1, which in turn suppresses insulin signaling. Early studies have shown that PTP1B overexpression inhibits phosphorylation of IR and IRS-1 leading to insulin resistance [7,8]. Recently, a correlation between insulin resistance states and expression levels of PTP1B in muscle and adipose tissues in humans has been also reported [9–11]. On the other hand, PTP1B knockout mice display an enhanced sensitivity to insulin, with increased tyrosine phosphorylation of the IR in the liver and muscle [12,13]. In addition, treatment with an antisense oligonucleotide specific for PTP1B results in improvement of hyperglycemia and insulin sensitivity in T2DM animal models [14,15]. Based on these findings, the inhibition of PTP1B

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has emerged as a potential therapeutic strategy to treat T2DM, and efforts on searching efficient PTP1B inhibitors are underway [16].

Pyrroloquinoline quinone (PQQ), a redox cofactor for bacterial dehydrogenases, has been implicated to be an important nutrient in mammals functioning as a potent growth factor [17]. PQQ is present in plants, animal tissues, and various foods [17–19]. More recently, we found that PQQ effectively inhibits PTP1B by oxidation of the catalytic cysteine through its redox cycling activity [20]. In this study, we demonstrate that PQQ stimulates insulin signaling through the inhibition of PTP1B activity in mouse C2C12 myotubes. Moreover, we evaluated the anti-diabetic effect of PQQ in KK-A^y mice, an animal model of T2DM.

2. Materials and methods

2.1. Materials

PQQ disodium salt was provided by Mitsubishi Gas Chemical Company (Tokyo, Japan). FBS, horse serum, and 2-[*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]-2-deoxy-D-glucose (2-NBDG) were obtained from Life Technologies (Carlsbad, CA, USA). Sodium orthovanadate (Na_3VO_4), ImmunoStar LD chemiluminescence detection kit, and sterilized 0.5% (*w/v*) methyl cellulose 400cP solution (0.5% MC) were purchased from Wako Pure Chemical (Osaka, Japan). DMEM, penicillin, and streptomycin were purchased from Nacalai Tesque (Kyoto, Japan). Insulin was obtained from Sigma–Aldrich (St. Louis, MO, USA).

2.2. Cell culture

The mouse myoblast cell line (C2C12) was obtained from Riken Cell Bank (Tsukuba, Japan) and cultured in DMEM (4.5 g/L glucose) supplemented with 10% FBS, 100 units/mL streptomycin, and 100 μg/mL penicillin at 37 °C in a humidified 5% CO_2 atmosphere. Myoblasts were induced to differentiate into myotubes 24 h after reaching confluency by changing the differentiation medium to DMEM supplemented with 1% horse serum, 100 units/mL streptomycin, and 100 μg/mL penicillin. The differentiation medium was changed every 48 h, and the myotubes (at days 10 and 11) were used for subsequent experiments. After the cells were starved in serum-free DMEM (1.0 g/L glucose) for 24 h, the myotubes were treated with PQQ in serum-free medium. After the treatment, the cells were washed twice with ice-cold PBS. Total cell lysates were prepared as reported previously [20].

2.3. Measurement of cellular PTP1B activity

Cellular enzymatic activity of PTP1B was assayed using tyrosine-phosphorylated EGFR residues 988–998 (Santa Cruz Biotechnology, CA, USA) as the substrate according to the published procedure [20]. The released phosphate from the phosphopeptide substrate was quantified using malachite green reagent (Biomol green reagent, Enzo Life Sciences, NY, USA).

2.4. SDS–PAGE and immunoblot analysis

SDS–PAGE and immunoblot analysis were done as described previously [20] with minor modification. To enhance the immunoreactions for phosphorylated proteins, we used an immunoreaction enhancer solution, Can Get Signal solution 1 and 2 (Toyobo, Osaka, Japan) to dilute primary and secondary antibodies, respectively. An ECL kit and LAS-4000 system (Fujifilm, Tokyo, Japan) were used for chemiluminescence detection. The following antibodies were used: anti-β-actin, anti-goat IgG-HRP, anti-mouse IgG-HRP, anti-IRS-1, anti-IRβ, and anti-PTP1B (Santa Cruz Biotechnology), anti-phospho-IRβ (Tyr1146), anti-rabbit IgG-HRP, anti-IRS-1 (pY^{612}), anti-phospho-Akt (Ser437), anti-phospho-Erk1/2 (Thr202/Tyr204), anti-Akt, anti-Erk1/2, and anti-GLUT4 (Cell Signaling Technology, Danvers, MA, USA).

2.5. Preparation of the plasma membrane fraction

The plasma membrane fraction was prepared according to the method of Nishiumi and Ashida [21] with some modifications. After treatment with insulin and PQQ, the cells were washed twice with cold PBS and homogenized with buffer A (50 mM Tris, pH 8.0, 0.1% Nonidet P-40, 0.5 mM DTT) containing 1 × Protease inhibitor cocktail and 1 × Phosphatase inhibitor cocktail (Nacalai Tesque) using a microtube pestle (five strokes) and 27-gauge syringe needle (five passages). The homogenate was centrifuged at 900*g* for 10 min at 4 °C, and the pellet was suspended again in buffer A. After centrifugation at 900*g* for 10 min at 4 °C, the pellet was washed with Nonidet P-40-free buffer A and spun at 900*g* for 10 min at 4 °C. The precipitate obtained was resuspended with buffer A containing 1% Nonidet P-40, and inhibitors and stood on ice for 1 h with occasional mixing. This suspension was centrifuged at 16,000*g* for 20 min at 4 °C, and the supernatant was used as the plasma membrane fraction.

2.6. Glucose uptake

The glucose uptake rate was measured by adding a fluorescent D-glucose analog, 2-NBDG, according to the published procedure [22] with some modification. C2C12 cells plated in the 96-well plate were incubated with 0–1000 nM PQQ for 5 h or 100 nM insulin for 30 min. After washing with glucose-free DMEM, 2-NDBG was added at a final concentration of 50 μM in glucose-free DMEM for another 20 min. The medium was then removed, and the cells were washed three times with cold PBS. The intensity of fluorescence was measured at ex/em 485/535 nm using a microplate fluorometer. Nonspecific uptake was determined in the presence of 10 μM cytochalasin B and was subtracted from all values.

2.7. Animal experiments

Animal experiments were conducted in accordance with the Guide for the Care and Use of Laboratory Animals of the Healthcare Products Development Center of KYOWA HAKKO BIO CO., Ltd. and were approved by the Animal Care Committee of the same Center. Six week-old male type 2 diabetic KK-A^y/TaJcl (KK-A^y) mice were purchased from Clea Japan (Tokyo, Japan). Animals were housed under a 12-h light/12-h dark cycle (lights on at 7 A.M.) with free access to water and rodent chow (CE-2, Clea Japan). After 1 week of environmental acclimation, diabetic mice were divided into 3 groups and given either 0.5% MC (vehicle, $n = 6$), 0.5 mg/mL PQQ disodium salt-0.5% MC (5 mg/kg/day PQQ, $n = 7$), or 2.0 mg/mL PQQ disodium salt-0.5% MC (20 mg/kg/day PQQ, $n = 5$) once a day *via* oral gavage (10 mL/kg body weight). Fasting blood glucose were measured before (Day-1) and after (Day 15) after the treatment. After the administration of PQQ for 14 days, an oral glucose tolerance test (OGTT) was performed. The KK-A^y mice were fasted for 18 h, followed by oral administration of 10% (*w/v*) glucose aqueous solution at a dose of 1 g/kg body weight. Blood was collected from tail veins at 0, 30, 60, and 120 min after the glucose administration. Blood glucose levels were measured using a Medisafe Reader GR-101 (Terumo Corporation, Tokyo, Japan). The area under the curve (AUC) between 0 and 120 min was calculated by the trapezoidal rule. Serum insulin concentrations were determined using a mouse insulin ELISA kit S-type (Shibayagi Co., Ltd, Gunma, Japan).

2.8. Statistical analysis

The statistical analysis was performed using the Dunnet test, except PTP1B activity assay (the two-tailed multiple *t*-test with Bonferroni correction). $P < 0.05$ was considered as statistically significant.

3. Results

3.1. PQQ inhibits PTP1B activity in C2C12 myotubes

We assessed cellular PTP1B activity in PQQ-treated C2C12 myotubes using a phosphorylated peptide substrate. As demonstrated in Fig. 1A, the exposure of C2C12 myotubes to PQQ for 5 h showed a dose-dependent inhibition of cellular PTP1B activity as well as Na_3VO_4, a specific PTP1B inhibitor. Meanwhile, we observed that PQQ had no effect on the expression level of PTP1B (data not shown). The PTP1B activity was significantly inhibited after the treatment with 500 nM PQQ for 1 h (Fig. 1B). Thus, these results suggest that PQQ can stimulate insulin signaling by inhibiting PTP1B activity.

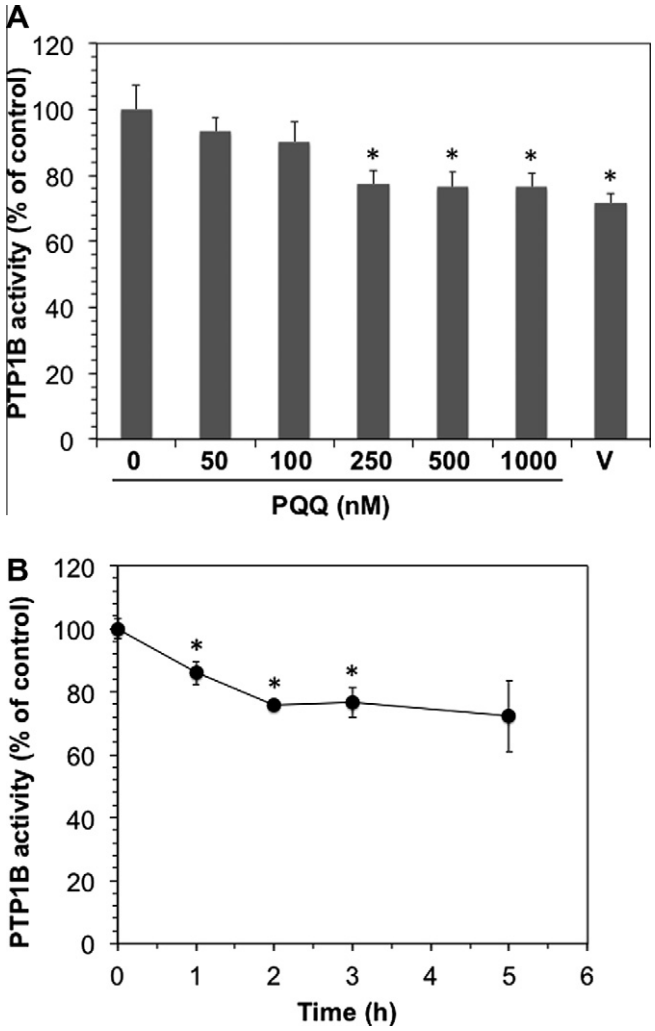

Fig. 1. Inhibition of PTP1B activity in C2C12 myotubes by PQQ treatment. (A) Concentration-dependent inhibition of PTP1B by PQQ treatment. C2C12 myotubes were treated with the indicated concentrations of PQQ or 200 μM Na_3VO_4 (V) in serum-free DMEM for 3 h, and then PTP1B activity was measured. The results shown are means ± SD ($n = 3$). $^*P < 0.05$ vs untreated control cells. (B) Time-dependent inhibition of cellular PTP1B by PQQ. C2C12 myotubes were treated with 500 nM PQQ for the indicated time in serum-free DMEM. The results shown are means ± SD ($n = 3$–5).

3.2. PQQ activates insulin signaling in C2C12 myotubes in an insulin-independent manner

The inhibition of PTP1B has been reported to evoke a ligand-independent phosphorylation of IR and activate downstream insulin signaling [23,24]. Inactivation of PTP1B would shift the tyrosine phosphorylation/dephosphorylation equilibrium towards phosphorylation since much lower tyrosine kinase activity will now suffice to cause IR phosphorylation and activation in the absence of the insulin. Hence, we investigated whether the treatment of C2C12 myotubes with PQQ induced a ligand-independent activation of insulin signaling by immunoblotting. We initially confirmed that PQQ showed no cytotoxicity at a concentration up to 100 μM over a period of 24 h (data not shown). As shown in Fig. 2A, the exposure of C2C12 myotubes to PQQ at concentrations from 50 to 1000 nM for 5 h caused a significant phosphorylation of IR at Tyr-1146, one of the prominent autophosphorylation sites. The insulin-independent phosphorylation of IR was significantly increased after the treatment with PQQ for 1 h (Fig. 2B). Furthermore, the PQQ-inducible tyrosine phosphorylation of IR was followed by the activation of downstream molecules of insulin signaling such as IRS-1, Akt, and Erk 1/2 (Fig. 2A and B). Meanwhile, there was no change in protein levels of IR, IRS-1, Akt, and Erk 1/2. These results suggest that PQQ can induce an insulin-independent increase in glucose uptake by activating insulin signaling.

3.3. PQQ promotes glucose uptake through GLUT4 in C2C12 myotubes in an insulin-independent manner

We next investigated whether PQQ-inducible activation of insulin signaling increases glucose uptake through the translocation of GLUT4 in C2C12 myotubes. We exposed C2C12 myotubes to PQQ (100 and 1000 nM) for 5 h in the absence of insulin and then GLUT4 levels in the isolated plasma membrane fraction were determined by immunoblotting. As shown in Fig. 3A, the treatment with PQQ significantly increased the amount of GLUT4 in the plasma membrane fraction to a similar extent as insulin, whereas the GLUT4 levels in whole cell lysates were not changed in PQQ-treated C2C12 cells as compared with vehicle-treated control.

To estimate the glucose uptake-promoting efficiency of PQQ, we measured the cellular uptake of a fluorescent D-glucose derivative, 2-NBDG, in PQQ-exposed C2C12 myotubes. As shown in Fig. 3B, we observed that the treatment with PQQ for 5 h at concentrations from 100 to 1000 nM significantly enhanced glucose uptake capacity of C2C12 myotubes, at levels similar to those of insulin-treated cells. The maximum increase in the 2-NBDG uptake was approximately 148% at 1000 nM PQQ. Thus, our findings suggest that PQQ may improve glucose metabolism by stimulating insulin signaling *in vivo*.

3.4. PQQ treatment ameliorates glucose tolerance in diabetic KK-A^y mice

To evaluate the anti-diabetic effect of PQQ *in vivo*, we administered PQQ (5 and 20 mg/kg body weight/day) for 2 weeks to diabetic KK-A^y mice. There was no significant difference in the food intake behavior, the final body weight, and body-weight growth trend among the diabetic control mice and the PQQ-treated diabetic mice during the 2-week period of treatment (data not shown). After 2 weeks of PQQ treatment, the fasting blood glucose levels in PQQ-treated mice declined slightly (Fig. 4A). We performed OGTT, which is usually used to diagnose diabetes mellitus, using PQQ-administered KK-A^y mice to estimate the blood glucose-regulating effect of PQQ. As shown in Fig. 4B, in both vehicle- and PQQ-treated mice, the blood glucose levels after glucose loading

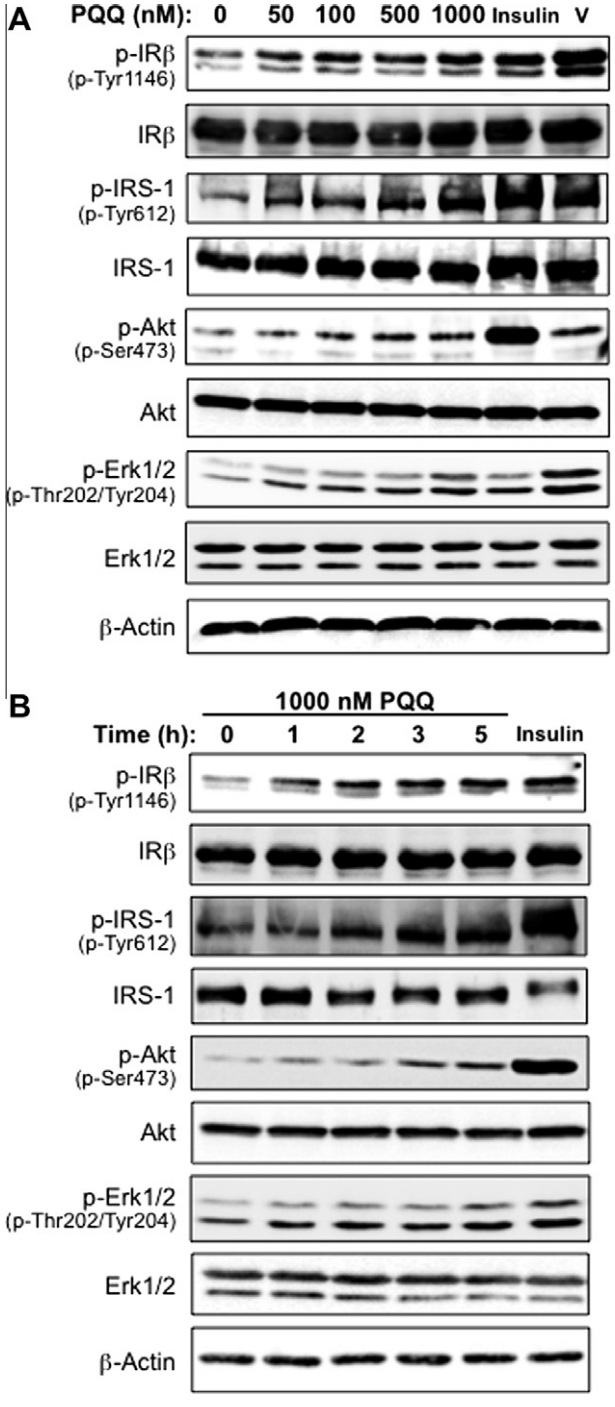

Fig. 2. Activation of insulin signaling during exposure of C2C12 myotubes to PQQ. (A) Concentration-dependent activation of insulin signaling by PQQ treatment. C2C12 myotubes were treated with the indicated concentrations of PQQ for 5 h or 100 nM insulin for 30 min or 200 μM Na_3VO_2 (V) for 3 h in serum-free DMEM. Then, phosphorylation of IRβ, IRS-1, Akt, and Erk 1/2 was determined by immunoblotting. (B) Time-dependent activation of insulin signaling by PQQ treatment. C2C12 myotubes were treated with 1000 nM PQQ for 0–5 h or 100 nM insulin for 30 min in serum-free DMEM. Then, phosphorylation of IRβ, IRS-1, Akt, and Erk 1/2 was determined by immunoblotting.

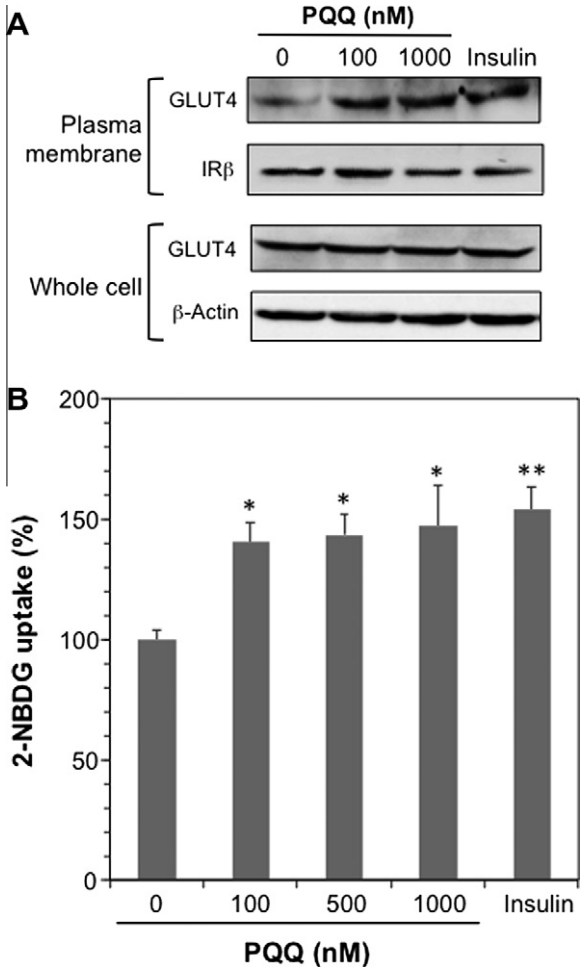

Fig. 3. Enhancement of GLUT4-mediated glucose uptake in C2C12 myotubes by the treatment with PQQ. (A) PQQ-inducible translocation of GLUT4. C2C12 myotubes were treated with the indicated concentrations of PQQ for 5 h or 100 nM insulin for 30 min in serum-free DMEM. Then, the plasma membrane fraction and whole protein were subjected to immunoblotting. (B) PQQ-inducible glucose uptake in C2C12 myotubes. C2C12 myotubes were treated with the indicated concentrations of PQQ for 5 h or 100 nM insulin for 30 min in serum-free DMEM. Then, glucose uptake was measured using a 2-NBDG fluorescent probe. The results shown are means ± SD ($n = 5$). $^{*}P < 0.05$, $^{**}P < 0.01$ vs vehicle-treated control.

sharply increased from 0 to 30 min and then gradually decreased. The peak blood glucose levels in the mice administered PQQ at 5 and 20 mg/kg/day were 274.9 ± 8.4 and 300.0 ± 24.1 mg/dL, respectively, and much lower than those in the vehicle-treated control mice (340.3 ± 10.8 mg/dL). The blood glucose levels at 60 and 120 min in PQQ-treated mice also showed significant decreases compared with control mice. Furthermore, PQQ treatment was found to significantly improve oral glucose tolerance as determined by analysis of AUC (Fig. 4C). On the other hand, there was no significant difference in serum insulin levels among the control mice and the PQQ-treated mice during periods of feeding (data not shown), indicating that the PQQ does not alter either insulin secretion or synthesis. These results suggest that PQQ improves glucose metabolism that is impaired in T2DM by stimulating the insulin signal.

4. Discussion

PQQ is a ubiquitous molecule that affects numerous physiological and biochemical processes and has been proved to be beneficial for growth and stress tolerance in both bacteria and higher organisms [17,25]. In recent years, PQQ has also been shown to play a role as an inducer for cellular redox signaling involved in regulating various physiological processes *via* its redox cycling property [20,25]. PQQ stably catalyzes continuous redox cycling as a cofactor and does so more efficiently than most other redox

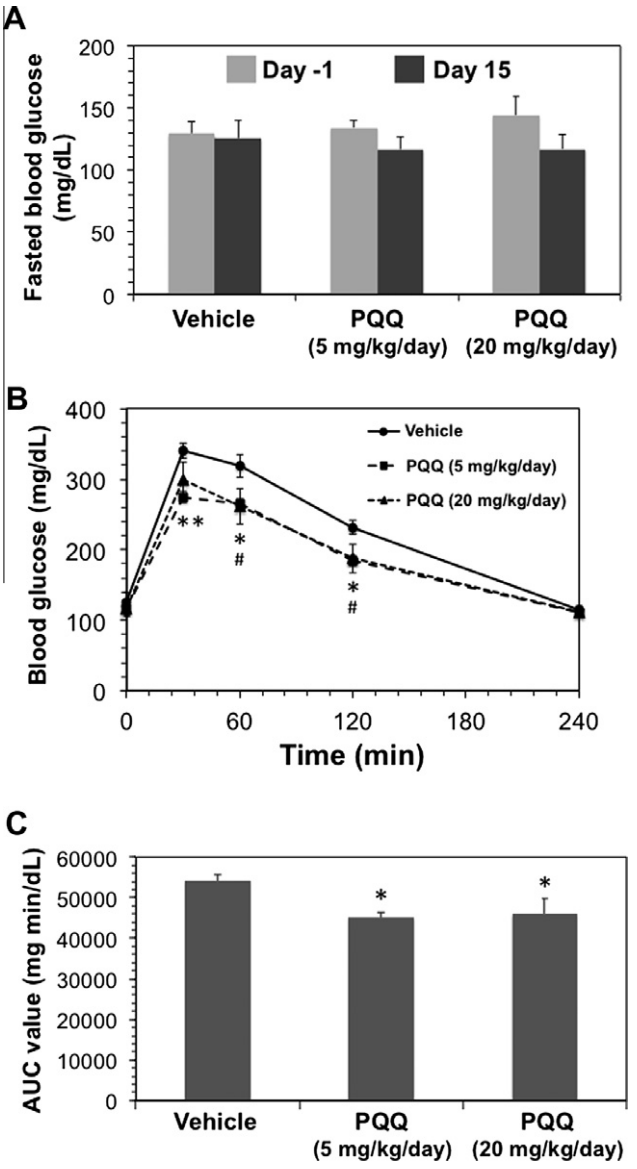

Fig. 4. Anti-diabetic effect of PQQ in diabetic KK-A^y mice. KK-A^y mice were administered vehicle alone ($n = 6$), 5 mg/kg/day PQQ ($n = 7$), or 20 mg/kg/day PQQ ($n = 5$) for 2 weeks. (A) KK-A^y mice were fasted for 18 h and fasting blood glucose levels were measured before (Day 1) and after (Day 15) each treatment. Data are shown as the mean ± SE. (B) Effect of PQQ on oral glucose tolerance. The fasted mice were injected with glucose (1 g/kg body weight) and blood glucose levels were determined at the time indicated. Data are shown as the mean ± SE. Asterisk and hash mark indicate a significant difference for the 5 mg/kg/day PQQ group and 20 mg/kg/day PQQ group, respectively, against the vehicle-treated control group ($^*P < 0.05$, $^{**}P < 0.01$, $^{\#}P < 0.05$). (C) The AUC values calculated from the result in (B). Data are shown as the mean ± SE. $^*P < 0.05$, $^{**}P < 0.01$ vs vehicle-treated control group.

active cofactors [17]. PQQ has been demonstrated to undergo one electron redox cycling, in the presence of reductants such as glutathione, to form the corresponding semiquinone radicals [17,20]. Thereafter, the generated semiquinones are reoxidized to the original quinones *via* the reduction of O_2 to O_2^-, which spontaneously or enzymatically dismutates to H_2O_2 and O_2. More recently, we found that PQQ efficiently inhibited PTP1B to oxidatively modify the catalytic cysteine (Cys-215) through its redox cycling activity to generate H_2O_2 [20]. Because of the characteristic environment surrounding the active site, the catalytic Cys-215 residue of PTP1B displays an extraordinary low pK_a (approx. 5.4), permitting its presence as an extremely reactive thiolate anion at a physiological pH [26]. Previous studies have revealed that the cellular redox state is involved in regulating the PTP1B activity through the reversible oxidization of the catalytic Cys-215 to Cys sulfenic acid (–SOH), which could be reduced with thiol reductants such as glutathione [26]. Furthermore, the generated Cys-SOH can be irreversibly over-oxidized to Cys sulfinic acid ($-SO_2H$) and Cys sulfonic acid ($-SO_3H$) in the presence of excess H_2O_2. Our recent study also demonstrated that the incubation of recombinant PTP1B with PQQ results in a dose-dependent formation of the active site Cys-SO_3H, which is suppressed by the addition of catalase [20]. Furthermore, we have shown that PQQ generated intracellular O_2^- and H_2O_2 *via* its redox cycling and inhibited PTP1B activity in an epithelial cell line A431. More importantly, we have revealed that PQQ evokes ligand-independent activation (tyrosine autophosphorylation) of epidermal growth factor receptor (EGFR) and its downstream signaling by inhibiting PTP1B, leading to increased cellular proliferation in A431 cells [20]. PTP1B has been demonstrated to have substrate-specific activity to dephosphorylate not only IR but also other receptor tyrosine kinases (RTKs) such as insulin-like growth factor-I receptor, EGFR, and nerve growth factor receptor, implicating the negative regulation of multiple growth factor signaling pathways [7,26]. Hence, inhibition of PTP1B is thought to elicit various physiological responses through enhanced RTKs-mediated signaling. Indeed, mice lacking PTP1B exhibit lower circulating insulin, glucose, and triglyceride levels, increased phosphorylation of the IR in liver and muscle after insulin injection, and improved insulin sensitivity in glucose and insulin tolerance tests [12,13,16]. Of note, PTP1B knockout mice have no obvious disease phenotype and normal fetal viability. Recent studies have also indicated that PTP1B expression and activity are significantly upregulated in the muscle and adipose tissue of obese and insulin-resistant humans and in rodent models of obesity and T2DM [9–11,16]. Based on these findings, we speculated that PQQ might stimulate insulin signaling and ameliorate impaired glucose metabolism in T2DM by inhibiting PTP1B. In the present study, as expected, we demonstrated that PQQ provokes the ligand-independent activation of insulin signaling by inhibiting cellular PTP1B and increases glucose uptake through the translocation of GLUT4 in C2C12 myotubes. Moreover, we confirmed that oral administration of PQQ improves glucose tolerance in diabetic KK-A^y mice.

Most of the small-molecule competitive PTP1B inhibitors mimic the tyrosine-phosphorylated IR, whereas the non-competitive inhibitors act through oxidation of the catalytic Cys-215 or by preventing the closure of the WPD loop [16]. Unfortunately, most of the synthesized inhibitors fail to succeed *in vivo* owing to unsatisfactory availability and side effects, whereas naturally occurring agents exert at least some of their effects on insulin signaling and glucose control *via* the inhibition of PTP1B [27]. However, the detailed molecular mechanisms underlying the inhibition of PTP1B by these natural compounds remain unclear. PQQ is present in various vegetables and beverages, especially in tea, natto (fermented soybeans), and fruits [17–19]. In addition, the nutritional and metabolic states of PQQ-supplemented mice and rats have already been well assessed [28]. Therefore, PQQ treatment can be expected to benefit T2DM subjects without adverse effects although its safe and effective dosage remains to be determined. In future, we must also evaluate the effects of PQQ treatment on IR phosphorylation levels in muscle and adipose tissue and on blood chemical parameters such as triglyceride, cholesterol, and free fatty acids levels *in vivo*. Further studies are needed to determine the effective therapeutic strategy for T2DM subjects.

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