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Communication

Pyrroloquinoline quinone increases the expression and activity of Sirt1 and -3 genes in HepG2 cells☆

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ABSTRACT

Sirtuin (Sirt) 1 and Sirt 3 are nicotinamide adenine dinucleotide ($^+$)-dependent protein deacetylases that are important to a number of mitochondrial-related functions; thus, identification of sirtuin activators is important. Herein, we hypothesize that pyrroloquinoline quinone (PQQ) can act as a Sirt1/Sirt3 activator. In HepG2 cell cultures, PQQ increased the expression of Sirt1 and Sirt3 gene, protein, and activity levels ($P < .05$). We also observed a significant increase in nicotinamide phosphoribosyltransferase gene expression (as early as 18 hours) and increased NAD^+ activity at 24 hours. In addition, targets of Sirt1 and Sirt3 (peroxisome proliferator–activated receptor γ coactivator 1α, nuclear respiratory factor 1 and 2, and mitochondrial transcription factor A) were increased at 48 hours. This is the first report that demonstrates PQQ as an activator of Sirt1 and Sirt3 expression and activity, making it an attractive therapeutic agent for the treatment of metabolic diseases and for healthy aging. Based on our study and the available data in vivo, PQQ has the potential to serve as a therapeutic nutraceutical, when enhancing mitochondrial function.

1. Introduction

Recent evidence suggests that environmental factors (eg, chemicals, physiologic stress, and nutrition) are important in the epigenetic regulation of metabolically active tissues. Indeed, the interaction between genes and the environment has emerged as a new frontier for the discovery of how networks of modified genes contribute to major pathologies. In this regard, the sirtuins have emerged as a group of mitochondrial nicotinamide adenine dinucleotide $(NAD)^+$-dependent protein deacetylases that act as cellular sensors in the regulation of a wide range of cellular processes [1].

Abbreviations: CR, caloric restriction; mRNA, messenger RNA; NAMPT, nicotinamide phosphoribosyltransferase; NRF-1, nuclear respiratory factor 1; NRF-2, nuclear respiratory factor 2; PGC-1α, peroxisome proliferator–activated receptor γ coactivator 1α; PQQ, pyrroloquinoline quinone; qRT-PCR, quantitative real-time reverse transcription–polymerase chain reaction; Tfam, mitochondrial transcription factor A.

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Among the 7 types of sirtuins, Sirt1 and Sirt3 have been most extensively investigated.

Our focus on Sirt1 and Sirt3 is fueled in part by the finding that Sirt3 activity is down-regulated upon exposure to a high-fat diet [1] and that Sirt1 plays a role in whole-body energy metabolism [2]. As specific examples, Sirt3-deficient mice display augmented mitochondrial protein hyperacetylation and accelerated development of the metabolic syndrome when fed a high-fat diet [1,3]. In addition to whole-body metabolism, Sirt1 appears important to pancreatic β-cell integrity and function [4], reducing myocardial hypertrophy [5] and neuroprotection [6]. Furthermore, both Sirt1 and Sirt3 are involved in the regulation of mitochondrial biogenesis [7] (Supplementary Fig. 2). Because sirtuins play beneficial roles in a number of pathophysiologic conditions, there is an increasing interest in identifying compounds, especially natural products that can modulate the activity of sirtuins or increase their expression levels [8]. To date, only few natural compounds (eg, resveratrol) have been found to activate Sirt1 [9].

Pyrroloquinoline quinone is an aromatic heterocyclic anionic orthoquinone found in plant foods, especially effective in neutralizing superoxide and hydroxyl radicals, two prominent causes of mitochondrial dysfunction. Most significantly, PQQ not only protects mitochondria from oxidative stress; it also promotes mitochondrial biogenesis [10]. Varying PQQ in the diets of mice and rats also results in changes in mitochondrial content and altered lipid metabolism [11]. However, many aspects of its mechanism of action remain unclear. Here, we hypothesize that PQQ can act as a Sirt1/Sirt3 activator. Thus, in the present study, we investigated whether PQQ has an effect on Sirt1/Sirt3 expression and activities. Using a HepG2 cell line, we also investigated whether the stimulation of the sirtuin pathway increased the mitochondrial function and biogenesis in the treated cells.

2. Methods and materials

2.1. Reagents

Pyrroloquinoline quinone disodium salt was a gift from Mitsubishi Gas and Chemical (Tokyo, Japan). Antibodies were purchased from Santa Cruz Biotechnology or Cell Signaling Technology.

2.2. Cell culture

Cells were cultured in Dulbecco's Modified Eagle's Medium media supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 100 mg/mL streptomycin. The cells were maintained in a humidified incubator at 37°C under 5% CO_2.

2.3. Quantitative real-time reverse transcription–polymerase chain reaction and immunoblotting

HepG2 cells (2.5×10^5 per well) were plated in 24 well plates. Twenty-four hours later, cells were incubated in control or PQQ-supplemented media for 48 hours, and total RNA was isolated from cells using E.Z.N.A Total RNA kit I (Omega Bio-Tek) according to the manufacturer's instructions. Complementary DNA was generated from 1 μg of RNA by reverse transcription (Applied Biosystems, Foster City, CA) Primer sequences are listed in the Supplementary Table 1. Relative gene expression was determined by quantitative real-time reverse transcription–polymerase chain reaction (qRT-PCR) and expressed relative to 18S.

For the immunoblotting, 1×10^6 cells were plated in 6 well plates. Twenty-four hours later, cells were incubated with either control Dulbecco's Modified Eagle's Medium or media supplemented with PQQ for 48 hours. Cells were washed with ice-cold phosphate-buffered saline and scraped into ice-cold radioimmunoprecipitation assay buffer lysis buffer or high-salt buffer [1] containing protease inhibitors (Sigma). The protein concentration in the cell lysate was determined using a Bradford assay. Total cell lysate (30 μg) was separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (Bio-Rad Ready Gels) under reducing conditions and transferred to nitrocellulose membrane. Bands were visualized by chemiluminescence using WesternSure ECL chemiluminescent substrate (LI-COR). Membranes were imaged using a LiCor C-digit Blot scanner, and blots were analyzed by ImageStudio software (LI-COR). Both actin and GAPDH were used as loading controls.

2.4. Sirtuin and NAD^+/nicotinamide adenine dinucleotide (NADH) activity assays

Sirt1 and Sirt3 activity assays were performed with slight modifications [1] using kits from Cayman Chemical Company (SIRT1 Direct Fluorescent Screening Assay Kit, catalog no. 10010401; SIRT3 Direct Fluorescent Screening Assay Kit, catalog no. 10011566). The NAD^+/NADH assay was performed according to the manufacturer's protocol using NAD/NADH Cell Based Assay Kit (catalog no. 600480) from Cayman Chemical Company.

2.5. Statistical analyses

All data are presented as the means ± SE. Statistical significance was determined by Student t test ($\alpha = .05$) or one-way analysis of variance using Tukey test ($\alpha = .05$).

3. Results and discussion

The present study is the first report to identify the effects of PQQ on sirtuins. Pyrroloquinoline quinone exposure (10-30 μM) significantly increased Sirt1 and Sirt3 gene and protein expression as well as activity and decreased overall protein acetylation in the human hepatocyte cell line, HepG2 (Fig. 1). The PQQ concentrations that were used were based on our previous observations [10]. Pyrroloquinoline quinone treatment did not alter membrane potential or cell viability (Supplementary Figs. 3 and 4). For comparison, to obtain similar results using resveratrol or piceatannol (a resveratrol metabolite) and a hepatocyte culture system, somewhat higher concentrations (>50 μM) are often needed [12]. With regard to PQQ, the need for micromolar amounts is most likely due to the reaction of PQQ with amino acids and proteins to form derivatives, primarily imidazolopyrroquinoline [13]. Imidazolopyrroquinoline does not influence mitochondriogenesis [10] but appears to be the dominant form of PQQ in

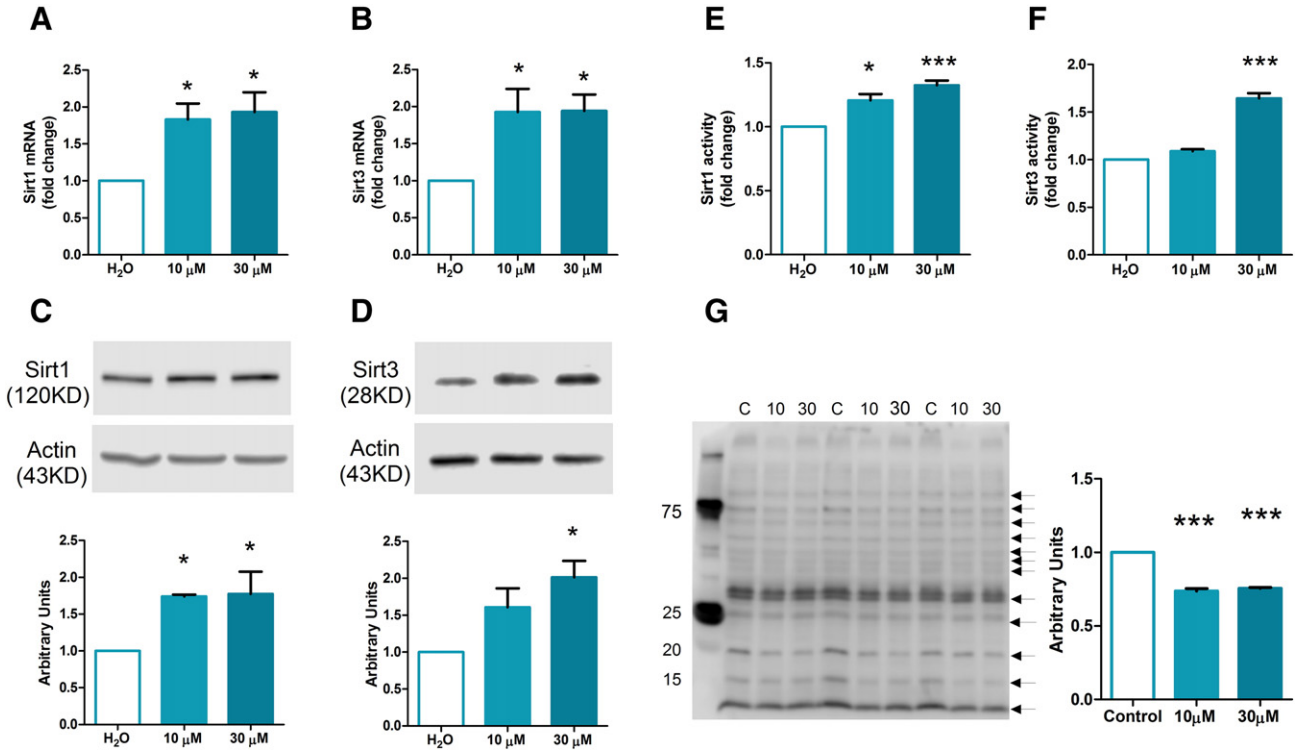

Fig. 1 – Pyrroloquinoline quinone increases Sirt1 and Sirt3 expression and activity in HepG2. HepG2 cells were exposed at 0, 10, and 30 μM PQQ for 48 hours. A and B, Expression of Sirt1 and Sirt3 mRNA was determined by qRT-PCR. 18S served as the reference gene. C and D, Whole-cell protein extracts were used to quantify the protein expression levels of Sirt1 and Sirt3. Western blotting analysis was carried out using equal amounts of protein. Values represent the ratio of Sirt1 or Sirt3 to actin, respectively (bar graph under the blot). E and F, Sirt1 and Sirt3 enzymatic activities were measured in protein extracts using commercially available kits. G, Protein acetylation was measured using anti-acetylated lysine antibody. Quantitative analysis of the blot is shown in a bar graph. All data in (A-G) are means ± SE. $^{*}P < .05$, $^{**}P < .01$, $^{***}P < .001$ vs untreated control (water), n = 3.

biological fluids, such as milk [13]. Given that imidazolo-pyrroquinoline can dissociate to PQQ with an apparent K_d constant of approximately 10^{-4}, that is, typical to those for other Schiff base and azomethine ylide derivatives, to insure nanomolar to sub micromolar concentrations of PQQ/$PQQH_2$ in cultures, micromolar concentrations of PQQ were added.

Increased Sirt1 and Sirt3 activity was also associated with increased nicotinamide phosphoribosyltransferase (NAMPT), NAD^+ activity levels, and mitochondrial biogenesis regulators (Fig. 2). Overexpression of NAMPT has been shown to result in increased Sirt2 activity [14]. We therefore carried out a time-dependent experiment at earlier time points, as we hypothesized that NAMPT needs to be up-regulated as early as possible to activate NAD^+ and subsequently increase sirtuin activity. Pyrroloquinoline quinone did not show any significant changes in NAMPT gene expression at 6 or 12 hours (data not shown). However, PQQ exposure leads to a significant NAMPT up-regulation at 18 hours ($P < .05$) (Fig. 2A) as well as an increase in NAD^+ activity at 24 hours ($P < .05$) (Fig. 2B).

Reduced levels of NAD^+ or Sirt1/3 expression or activity can lead to impaired metabolic function. In this regard, an accumulating body of evidence has shown that caloric restriction (CR) protects age-related metabolic dysfunctions and oxidative damage by increasing Sirt3-mediated deacetylation of several modulators. Interestingly, CR and fasting enhance NAD^+ levels in the liver mitochondria, possibly through the induction of NAMPT [15]. In contrast, decreased NAMPT levels have been observed in human adults with non–alcoholic fatty liver disease and cirrhosis [16,17]. Of therapeutic potential, sirtuins exhibit a beneficial phenotype resembling CR [18].

In our published study [10], we showed that PQQ increases peroxisome proliferator–activated receptor γ coactivator 1α (PGC-1α) messenger RNA (mRNA) and protein expression in Hepa 1-6 cell line. In HepG2 cells, PQQ also significantly increased the PGC-1α messenger RNA (Fig. 2C), although protein expression was only modestly increased (Supplementary Fig. 5A). In this regard, one limitation is that PGC-1α is expressed at very low levels in HepG2 cells [19,20]. Nevertheless, the patterns of change in PGC-1α and the related significant findings suggest a high degree of similarity between the observations in Hepa 1-6 and HepG2 cells. Although described as a master regulator of mitochondrial biogenesis and energy homeostasis, PGC-1α activity is tightly controlled by the metabolic sensors sirtuins, which directly affect its activity through deacetylation [21]. Both Sirt1 and Sirt3 produced a modest PGC-1α deacetylation (Supplementary Fig. 5B) in HepG2 cells. Clearly, more studies are

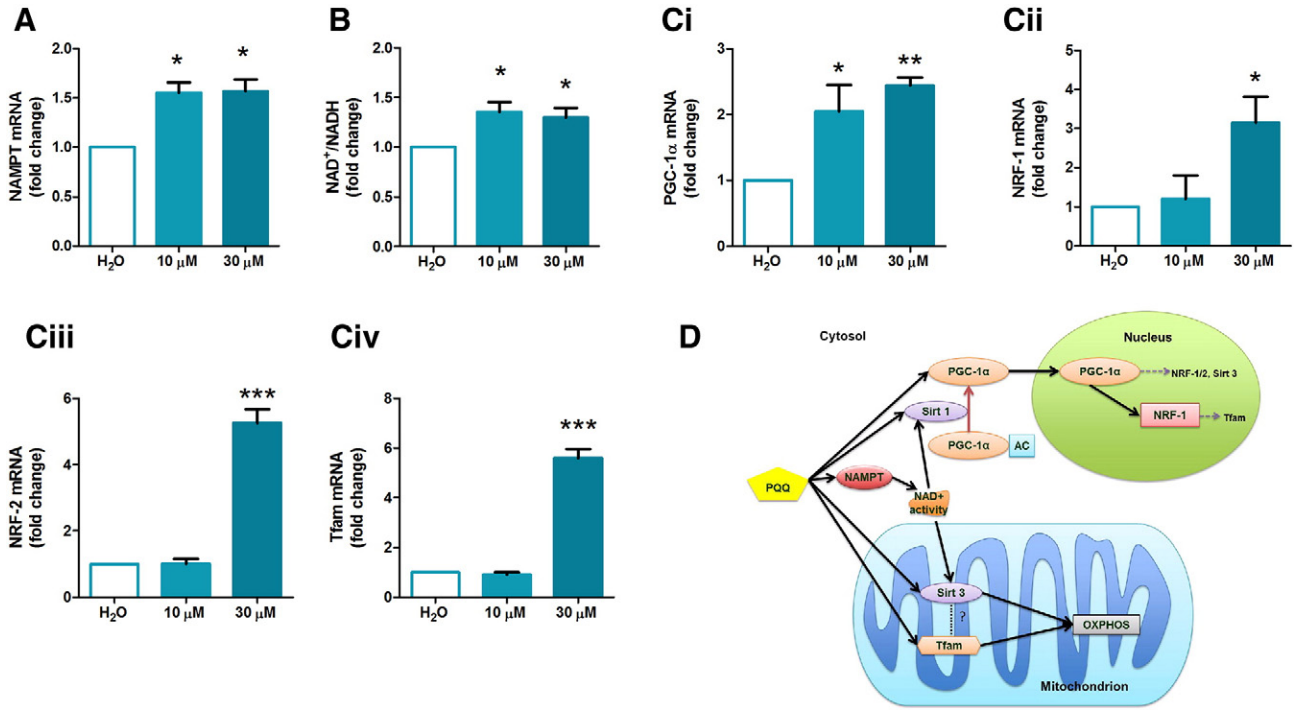

Fig. 2 – Effect of PQQ on regulatory factors. HepG2 cells were exposed for 18 or 24 or 48 hours to 10 or 30 μM of PQQ. A, Expression of NAMPT mRNA levels was determined by qRT-PCR after 18 hours treatment of PQQ. 18S served as the reference gene, n = 3. B, NAD^+/NADH ratio was measured after 24 hours of treatment with PQQ using the commercially available kit, n = 4. C, Messenger RNA expressions of (i) PGC-1α, (ii) NRF-1, (iii) NRF-2, and (iv) Tfam were quantified by qRT-PCR (n = 3). All data are expressed as the means ± SE. $^*P < .05$, $^{**}P < .01$, $^{***}P < .001$ vs untreated control (water), n = 3. D, Schematic depiction of a novel molecular pathway of PQQ.

needed to identify the involvement of other mechanisms, and, given the linkages of PGC-1α and Sirt1 and 3 to energy metabolism, in vivo experiments will be essential.

What is appreciated is that Sirt1 influences glucose, lipid, and cholesterol metabolism in the liver. Sirtuin 1 also activates transcription of nuclear and mitochondrial genes, encoding for proteins promoting mitochondria proliferation, oxidative phosphorylation, and energy production. Sirtuin 3 directly acts as an activator of proteins important for oxidative phosphorylation, tricarboxylic acid cycle, and fatty-acid oxidation; and it acts indirectly on PGC-1α and AMP-activated protein kinase [7]. Several of these mitochondrial-related events are also regulated by nuclear respiratory factors (NRFs) [22]. Consistent with the previous study using mouse Hepa 1-6 cells [10], PQQ also up-regulates the gene expression levels of mitochondrial regulators NRF-1, NRF-2, and mitochondrial transcription factor A (Tfam) in HepG2 (Fig. 2C). Several in vivo and in vitro studies implicate Tfam as an ideal target for regulatory pathways that control both mitochondrial DNA maintenance and transcriptional expression. Furthermore, NRF-1 binding sites are present in the promoters of several nuclear genes required for mitochondrial respiratory function. It is also important to note that NRF-1 targets are involved in the regulation of metabolic enzymes, components of signaling pathways, and gene products necessary for chromosome maintenance and nucleic acid metabolism [23]. A second nuclear factor designated as NRF-2 also plays an important regulatory function in respiratory chain expression [24] as well as the transcription factors [25], TFB1M and TFB2M [26]. Therefore, PQQ can improve the overall mitochondrial function.

With regard to PQQ, we conclude that PQQ induces the activation of both Sirt1 and Sirt3 in concert with the expression of PGC-1α and other respiratory factors involved in mitochondrial biogenesis. Although limited, the available data for humans suggest that ingestion of PQQ at 0.2 to 0.3 mg PQQ/kg body weight results in changes in urinary metabolites consistent with enhanced mitochondria-related functions [27]. Only 10 mg quantities or less of PQQ/kg of typical animal diets elicit mitochondrial-related responses, compared with the 20 to 30 times higher amounts needed for a resveratrol response [28]. Using rats fed chemical-defined diets devoid of PQQ, the addition of PQQ in amounts as low as 2 mg PQQ/kg diet improves energy utilization and lipid metabolism and protects against ischemia reperfusion injury [11]. Furthermore, mice fed diets devoid of PQQ display poor reproductive performance and compromised neonatal growth and survival [28]. An important feature is that PQQ is relatively soluble in water and its chemical stability making it attractive as a therapeutic agent.

In conclusion, we demonstrate that PQQ is an activator of Sirt1 and Sirt3 expression and activity, making it an attractive therapeutic agent for the treatment of metabolic diseases or for healthy aging (Supplementary 2). Recently, PQQ has shown to ameliorate streptozotocin-induced oxidative damage in the brain as well as the streptozotocin-induced

diabetes [29]. In addition, oral administration of PQQ improved impaired glucose tolerance in type 2 diabetic KK-A(y) mice [30]. Based on the work reported here and available data in vivo, PQQ has the potential to serve as a potential therapeutic nutraceutical, when enhancing mitochondrial function. Consequently, it should also be promising to examine further sirutin activation in models in vivo important to the understanding of metabolic syndrome and aging.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.nutres.2015.06.014.

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