Alpha Hope, via Molecular Hydrogen and Pyrroloquinoline Quinone, Dose-Dependently Increases Nrf2 and PGC-1α Levels in C2C12 Myoblasts

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Abstract: Alpha Hope is a dietary supplement containing pyrroloquinoline quinone and elemental magnesium, which produces molecular hydrogen (H₂ gas) when dissolved in water. We determined the impact of Alpha Hope on Nrf-2, peroxisome proliferator-activated receptor-gamma coactivator (PGC-1α), and citrate synthase (CS) activity in C2C12 myoblasts. Alpha Hope was titrated to three concentrations of pyrroloquinoline quinone (PQQ) (10 nM, 100 nM, 1000 nM) and treated on C2C12 for 48 h. Nrf-2 and PGC-1α levels were measured using Western blot analysis. CS activity was measured according to previously described methods. Treatment significantly increased Nrf-2 and PGC-1α protein levels in C2C12 myoblasts, with no change for CS. For Nrf-2, values for both the 100 nM (p = 0.046) and 1000 nM (p = 0.011) concentrations were higher than control. For PGC-1α, values for both the 100 nM (p = 0.039) and 1000 nM (p = 0.017) concentrations were higher than control. In a small human pilot study, subjects consumed the Alpha Hope product daily for four weeks, with no adverse effects, with some subjects noted as “responders” to treatment. Alpha Hope can significantly increase both Nrf-2 and PGC-1α in a concentration-dependent manner. Healthy men and women who ingest the product daily can do so without adverse effects.  

Keywords: PQQ; molecular hydrogen; dietary supplement; myoblasts; PGC-1α; Nrf-2

1. Introduction  

Pyrroloquinoline quinone (PQQ) is a quinone-containing, noncovalently bound redox cofactor, first described in 1964 as the “new prosthetic group” in glucose dehydrogenase from Bacterium anitratum [1]. As a redox cofactor, PQQ has some activity similar to that of the B vitamins; hence, it has been considered a member of the B complex group of vitamins. Investigators have hypothesized that humans might exhibit a PQQ deficiency and that it should be considered to have a potential nutritional requirement similar to vitamins [2].  

PQQ is found ubiquitously in plants and has several biological functions. These include promoting mitochondrial biogenesis [3] and sirtuin activity [4], with varied physiological outcomes pertaining to cognition [5] and neuroprotection [6,7]. It is considered to be neuroprotective in the central nervous system and peripheral nerves and helps enhance neuronal cell regeneration [8].  

PQQ is often cited as an antioxidant [9,10] and is thought to be more efficient in redox cycling assays than various other compounds including ascorbic acid, menadione, isoflavonoids, and polyphenolic compounds [2]. As a redox cofactor, it has the ability to...
catalyze continuous redox reactions [11]. Importantly, PQQ has health effects that extend beyond its activity and function as a redox cofactor [2].

For example, it has been reported that PQQ can increase the expression of the peroxisome proliferator-activated receptor-gamma coactivator (PGC-1α) [3]. PGC-1α is considered to be a master regulator of mitochondrial biogenesis. It plays a role in proper mitochondrial function, energy metabolism, and ATP production. [12]. Additionally, PGC-1α operates as a transcriptional coactivator to promote nuclear respiratory factors 1 and 2 (NRF-1 and NRF-2), which may regulate the expression of genes that contain antioxidant-response elements (AREs) in their promoter regions [13]. These cellular factors are essential to maintain energy balance and encourage antioxidant response genes. It is known that during the aging process, cellular mitochondria are thought to be one of the most impaired organelles. Therefore, the removal of mitochondria and the manufacturing of new mitochondria are critical for metabolic energy homeostasis [12]. By increasing PGC-1α and subsequent mitochondrial biogenesis, there may be an improvement in maintaining the health of cells with the removal and regeneration of mitochondria. With aging there is a decline in PGC-1α levels, which contributes to mitochondrial dysfunction.

Brain-derived neurotrophic factor (BDNF) is a protein and a member of the neurotrophin family and is an essential compound in plasticity related to learning and memory. Expression of BDNF is highly variable in healthy subjects. The changes observed in BDNF expression are associated with both normal and physical impairments during aging [14]. BDNF expression is also influenced by memory-related problems. BDNF plays key roles in the development, maintenance, and repair of the nervous system and has emerged as an important regulator of synaptic plasticity and learning and memory. There is an association between BDNF and PGC-1α. BDNF signaling has been shown to significantly increase mitochondrial biogenesis and has been demonstrated to upregulate PGC-1α expression [15].

PGC-1α also has many other tissue-specific tasks including adipogenesis, gluconeogenesis, thermogenesis, and cellular protection against degeneration [16]. Prior work in animals demonstrates favorable outcomes pertaining to resilience to symptoms of depression in animals that were genetically modified to overproduce PGC-1α in type II skeletal muscle fibers [17]. Moreover, it has been reported that PGC-1α is involved in the formation and maintenance of neuronal tissue [15]. This may be associated with changes in the levels of neurotrophins, such as BDNF, which may reduce symptoms of depression. PGC-1α can also promote the expression of nuclear factor erythroid 2–related factor 2 (Nrf-2) [2]. This activity from transcriptional factors is important for cellular equilibrium and to help regulate genes involved in antioxidant protection [18].

Nrf-2 is a transcription factor involved in regulating cellular resistance to oxidants and could be influenced by PQQ [19]. The significant mechanism in cellular defense is initiated by the phase II enzymes through stimulation of the Nrf-2-antioxidant response element (ARE) signaling pathway [20]. Nrf-2 is essential in regulating electrophile/antioxidant homeostasis. It provides support to the cell’s integrity and function, especially under conditions of oxidative stress. The Nrf-2/ARE signaling pathway helps to control the expression of over 200 genes that participate in antioxidation and detoxification [20]. Current research shows that Nrf-2 operates in a dominant way to promote neuroprotection because of its antioxidant response [21]. Collectively, it is believed that PQQ can impact both PGC-1α and Nrf-2, while also influencing citrate synthase activity, a marker for mitochondrial content in cells and tissues [3,22]. Indeed, PQQ has been demonstrated to increase citrate synthase activity, which was blocked in PGC-1α knockdown using small interfering RNA [3].

Molecular hydrogen (H2 gas) has recently been recognized as a “novel” antioxidant for preventive and therapeutic health benefits [23]. The use of hydrogen has been reported in numerous papers showing a range of beneficial biological effects including anti-inflammatory and antioxidation properties, as well as attenuating age-related disorders and reducing fatigue [24,25]. The manufacturing and production of H2 are well known. For therapeutic use, a commonly used commercially available method is in the form of a
tablet that contains elemental magnesium, which, when placed into water, produces H$_2$ gas [24,26]. H$_2$ has been described as a regulator of Nrf2-mediated redox signaling and has therapeutic effects within the mitochondria [27]. In addition, H$_2$ has been shown to induce the expression of the PGC-1α gene and afterward stimulation of the PPARα pathway which regulates fibroblast growth factor-21 (FGF21) [28].

The present study investigated the in vitro and in vivo impact of Alpha Hope. This is a proprietary and novel H$_2$-producing dietary supplement that also contains PQQ. In the in vitro study, the concentrations of PGC-1α and Nrf-2 and the citrate synthase activity in C2C12 myoblasts following treatment with Alpha Hope at three different concentrations were measured. We hypothesized that values for all outcomes would be increased in a concentration-dependent manner. For the in vivo study, we evaluated safety/tolerability and various blood biomarkers following Alpha Hope supplementation in healthy subjects. We hypothesized that Alpha Hope would be well tolerated and improve these markers. To our knowledge, this is the first time the impact of simultaneous administration of H$_2$ and PQQ has been evaluated in vivo or in vitro.

2. Materials and Methods

2.1. In Vitro Study

For the in vitro study, we used Alpha Hope (CalerieHealth, Irvine, CA, USA), titrated to three concentrations as stated below. The Alpha Hope supplement contains a total of 10 mg PQQ and 80 mg of magnesium per tablet. For tablets, the H$_2$ is produced by the active ingredient, elemental magnesium (80 mg), which reacts with water to produce H$_2$ gas and magnesium hydroxide according to the following reaction: Mg + 2H$_2$O $\rightarrow$ H$_2$ (g) + Mg(OH)$_2$ [20,29]. The form of PQQ used in Alpha Hope is BioPQQ, which has Generally Recognized as Safe (GRAS) designation. Hydrogen water also has GRAS status through the FDA. C2C12 myoblasts were purchased from the American Type Culture Collection (ATCC).

The Alpha Hope supplement was dissolved in cell culture media containing Dulbecco’s Modified Eagle Medium (DMEM) with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin. Three concentrations were used, titrated to PQQ at 1000 nM, 100 nM, and 10 nM, and treated on C2C12 for 48 h, with fresh media changed every 24 h. This titration range was chosen according to Nakano et al. [30]. The initial concentration of H$_2$ in the media was expected to be less than 50 µM at the highest concentration and less than 1 µM at the lowest concentration and return to baseline (<0.5 nM) within 30 min. Control cells did not receive the CalerieHealth Alpha Hope supplement.

After 48 h of treatment, cells were lysed with RIPA buffer, collected, and stored at -80°C until analyzed. PGC-1α and Nrf-2 levels were measured using Western blot analysis. Western blots were conducted on cell samples to analyze peroxisome proliferator-activated receptor gamma-coactivator 1a (PGC-1α, GTX37356, GeneTex) and nuclear factor erythroid 2-related factor 2 (Nrf-2; GTX103322, GeneTex) levels. Each membrane was stained with Ponceau and was used as the loading and transfer control. A chemiluminescent system was used to visualize marked proteins (GE Healthcare Life Sciences, Pittsburgh, PA, USA). Images were taken and analyzed with iBright Imaging Systems (Thermofisher Scientific, Waltham, MA, USA). Citrate synthase (CS) activity, which serves as a marker for mitochondrial content, was measured according to the methods described by Zhang et al. [31]. Buffer for the CS activity assay included 100 mM Tris at pH = 8.0, 0.1% (v/v) Triton X-100, 100 mM DTNB, and 300 mM acetyl-CoA with 1 mg of cell lysis. Baseline activity was measured for 2 min, and reactions were started by adding the final concentration of 0.5 mM oxaloacetic acid and measured for 3 min at 412 nm using a Synergy H1 hybrid plate reader (BioTek Instruments, Inc., Winooski, VT, USA).

For the in vivo study, a small-scale human pilot study was conducted on 14 healthy subjects (11 women and 3 men (56 ± 9 years)). Subjects were randomized to Alpha Hope or placebo tablets (two/day) for a period of four weeks in a double-blinded, cross-over design. We evaluated the influence of Alpha Hope on plasma levels of brain-derived neurotrophic...
factor (BDNF), fibroblast growth factor-21 (FGF21), and irisin. We also administered some basic cognitive functional tests (i.e., digit symbol substitution, AX-continuous performance test, and Go/No-Go Test) as described previously [32].

2.2. Statistical Analyses

All statistical tests for the in vitro data were carried out using GraphPad Prism 9. Tests for the human pilot study were carried out using JMP Statistical software (Cary, NC, USA). Data are expressed as mean ± SEM and were analyzed by one-way ANOVA with differences between treatment concentrations detected by Dunnett’s post hoc and deemed significant when \( p < 0.05 \).

3. Results

3.1. In Vitro Study

In the in vitro study, treatment with Alpha Hope for 48 h significantly increased Nrf-2 and PGC-1\(\alpha\) levels in C2C12 cells in a dose-dependent manner (Figure 1A,B). Specifically, although Nrf-2 values for the 10 nM PQQ concentration were not different from the control \( (p = 0.417) \), the Nrf-2 values for both the 100 nM \( (p = 0.046) \) and 1000 nM \( (p = 0.011) \) concentrations were dose-dependently higher than the control (see Figure 1A). Likewise, PGC-1\(\alpha\) values for the 10 nM PQQ concentration were not different from the control \( (p = 0.727) \), but the PGC-1\(\alpha\) values for both the 100 nM \( (p = 0.039) \) and 1000 nM \( (p = 0.017) \) concentrations were higher than the control (see Figure 1B).

![Figure 1. Effects of different concentrations of Alpha Hope on C2C12 myoblasts after 48 h. Nrf2 (A) and PGC-1\(\alpha\) (B).](image)

Additionally, to determine mitochondrial function, citrate synthase activity was also measured. However, despite a potential dose-dependent trend, a 48 h treatment of Alpha Hope failed to significantly increase citrate synthase activity in C2C12 cells \( (p > 0.05) \); Figure 2).
3.2. In Vivo Human Pilot Study

The preliminary human pilot study (n = 14) demonstrated the safety of the Alpha Hope supplement, as evidenced by no self-reported adverse effects. Additionally, resting heart rate and blood pressure were not influenced (p > 0.05) by Alpha Hope, and values remained within normal ranges. Furthermore, the supplement was well tolerated amongst all subjects with no dropouts due to treatment. There were no statistically significant impairments or improvements in the cognitive tasks, or in the plasma markers (i.e., brain-derived neurotrophic factor (BDNF), fibroblast growth factor-21 (FGF-21), and irisin). However, sub-analysis revealed that select subjects appeared to be “responders” to treatment, in terms of blood irisin, brain-derived neurotrophic factor, and FGF-21. For example, using Alpha Hope, two subjects experienced an increase in irisin (>25%), three subjects experienced an increase in BDNF (>50%), and two subjects experienced an increase in FGF-21 (>50%).

4. Discussion

The present in vitro study demonstrates that the Alpha Hope dietary supplement providing H2 and PQQ can increase both Nrf-2 and PGC-1α levels in C2C12 myoblasts. There are various methods to administer H2, but regardless of the method of administration, the bioavailability of H2 is high [33]. Although a potential dose-dependent trend in citrate synthase activity was observed, statistical significance was not reached. However, a longer treatment duration (e.g., more than 48 h) and/or a higher concentration may have reached statistical significance. The limited exposure time is particularly true for molecular hydrogen, as its concentration would have fallen below the micromolar/therapeutic threshold within less than 30 min [34]. Moreover, it is estimated that an H2 concentration of at least several micromolar may be needed to induce a biological effect. In this in vitro study, the highest concentration of H2 was estimated to be around 50 µM, but the lowest concentration may not have reached 1 µM. Therefore, our results may corroborate this assumption of the minimal cellular concentration that is needed to induce a therapeutic effect.

In addition to concentration, the duration of exposure is also an important consideration. It is estimated that the concentration of H2 would have decreased to baseline (atmospheric) levels (i.e., <0.5 nM) within 30 min. However, such limited exposure appears to be enough time to induce biological effects such as induction of Nrf2. Moreover, repeated intermittent exposure to H2 (15 min per hour) was more effective than constant exposure in a rat model of Parkinson’s disease [35]. Finally, it should be noted that the C2C12 myoblasts were not exposed to cellular stress (e.g., radiation, H2O2, drugs, environmental pollutants,
etc.), which is often required in order to unveil the cytoprotective effects of molecular hydrogen [20]. Indeed, if redox and metabolic homeostasis is already optimal, then we should not expect (or desire) significant changes from baseline. Future research is needed to determine the potential independent contribution of both PQQ and molecular hydrogen, particularly during times of cellular stress.

In the in vivo human study, it was demonstrated that Alpha Hope is safe and well tolerated in otherwise healthy subjects. As expected, Alpha Hope did not impair cognitive performance, and subjects largely “maxed out” the tests, ostensibly obfuscating any potential improvements from the Alpha Hope supplement. Similarly, as expected in these healthy, non-diseased subjects, Alpha Hope did not ubiquitously increase plasma markers (BDNF, FGF-21, irisin). However, some subjects did demonstrate significant increases in levels of irisin (>25%), BDNF (>50%), and FGF-21 (>50%). Still, it is impossible to draw definitive or generalizable conclusions because of the small sample size. Indeed, some of these changes may be attributed to known confounders such as a regression to the mean or other statistical anomalies.

Nevertheless, it is not unreasonable that Alpha Hope contributed to these changes since both molecular hydrogen and PQQ can influence these biomolecules [36]. Moreover, a stronger effect may be noticed with subjects who are further away from normal homeostasis such as those under increased stress, sleep deprivation [37], or intense physical exercise [33] or an older population. Future studies should take these ideas into consideration. This is also true for the in vitro study, in which favorable effects on Nrf2 and PGC1α were observed despite it occurring in non-stressed myoblast cells. However, had the cells been stressed, e.g., impaired mitochondrial function, there may have been greater increases, as well as an increase in citrate synthase activity, a marker of mitochondrial function.

Mitochondrial function is essential in terms of overall health, and impairment is associated with multiple diseases and metabolic disorders. In addition, mitochondrial function is diminished during aging, with reduced levels of PGC-1α contributing to this problem [12]. The removal of old mitochondria and the manufacturing of new mitochondria are critical for cellular metabolism [12].

Early studies revealed that dietary PQQ had a positive effect on mitochondrial biogenesis in vivo, although the mechanism of action was not determined [11]. Prior work showed that PQQ promoted PGC-1α activity, which is considered to be a mechanism for the regulation of mitochondrial biogenesis [38]. PQQ has been shown to improve the mitochondrial tricarboxylic acid (TCA) cycle, which subsequently stimulates mitochondrial biogenesis [3]. Providing a small milligram dose of PQQ per kg of the diet or even micromolar amounts in vivo stimulates mitochondrial biogenesis [3]. Improved mitochondrial bioenergetics is an important factor in the body’s utilization and production of energy, optimizing longevity, and protecting against oxidative stress induced by reactive oxygen and nitrogen species.

PQQ as an antioxidant could be incorporated into the mitochondrial inner membrane and matrix [11]. Reactive oxygen and nitrogen species serve as useful signaling molecules but may also cause damage to cells when produced in excess. Therefore, a desirable objective is to decrease the excess production of these oxidants and/or stimulate the intracellular oxidative defense capacity or use exogenous antioxidants. PQQ has been shown to have an antioxidant effect, possibly mediated through phosphatidylinositol-3-kinase (PI3K)/Akt signaling [39].

While PQQ may work through its impact on PGC-1α, it can also impact Nrf-2, which is essential to help the cell defend against oxidative stress. As reported in the present study and elsewhere [40], treatment with PQQ activates Nrf-2 and upregulates mRNA expression of Nrf-2. The Nrf2/Keap-1 complex is present within the cytoplasm, and once activated, Nrf-2 separates from the complex to move into the nucleus. It then initiates the expression of genes encoding different antioxidant molecules [41]. Nrf-2 has been shown to provide protection against oxidative stress [19]. Although PGC-1α is clearly the regulator of mitochondrial biogenesis, some investigators believe that Nrf2 activation is involved [42]. Although Nrf2 regulates the expression of many antioxidant genes, there is also research
that shows that there is a regulatory loop that includes the engagement of PGC-1α and Nrf2. For example, there are certain disease conditions that inhibit mitochondrial biogenesis, and it is suggested that focusing on ways to elevate Nrf2 and PGC-1α may have a beneficial effect to slow down these pathological conditions [42]. Activation of Nrf2-dependent mitochondrial biogenesis has been demonstrated to occur upon moderate physical exercise and can improve specific health conditions. Indeed, one study showed that moderate physical exercise promotes Nrf2-dependent mitochondrial biogenesis in muscles [43].

PGC-1α is frequently expressed in organs such as the brain where there is an elevated demand for energy. If PGC-1α is depleted in the brain, it causes neurons to degenerate by causing mitochondrial dysfunction [44]. Importantly, regarding the effect of PQQ on brain function, a 12-week randomized, placebo-controlled study was performed with 41 healthy older subjects. They were provided with a daily dose of 20 mg of PQQ or placebo [9]. Subjects were tested for cognitive function and blood flow to the brain using near-infrared spectrometry. The study showed that PQQ increased cerebral blood flow. In addition, after 12 weeks, the PQQ group had improvements in working memory and attention to tasks.

Another study was performed measuring regional cerebral blood flow (rCBF) and oxygen metabolism in the prefrontal cortex (PFC) [45]. Measurements were taken at baseline and then after administering 20 mg PQQ in 20 healthy older subjects. The investigators used time-resolved near-infrared spectroscopy (tNIRS). Hemoglobin (Hb) concentration and absolute tissue oxygen saturation (SO₂) in the bilateral PFC were examined. The PQQ group had significant increases in the hemoglobin and total hemoglobin levels in the right PFC (p < 0.05). Additionally, the reduction in the SpO₂ levels in the PFC was more prominent than that in the placebo group (p < 0.05). These results suggest that PQQ causes increased activity in the right PFC associated with increases in rCBF and oxygen metabolism, resulting in enhanced cognitive function. The investigators suggested that this increase in brain blood flow determined as cerebral oxygenation most likely was the reason for the improvement in enhanced cognitive function.

Interestingly, molecular hydrogen may also provide similar benefits in increasing blood flow and prefrontal cortex activation. For example, 20 min of administration of molecular hydrogen resulted in increased PFC activation, which was associated with the alleviation of physical fatigue and reduced perceived exertion during high-intensity exercise in healthy young adults [46]. Moreover, hydrogen water has been associated with increased oxygen saturation and improved exercise tolerance [47]. These results may be partially explained by the favorable effects of molecular hydrogen on erythrocytes. For example, it was demonstrated that in rats, the administration of molecular hydrogen resulted in an increase in erythrocyte ATP production and levels of 2,3 bisphosphoglycerate, which consequently improved microcirculation and oxygen transport [48].

Importantly, molecular hydrogen, as the smallest molecule and having a neutral charge, can easily penetrate cellular mitochondrial and nuclear membranes. Its molecular stability prevents it from reacting with and neutralizing important signaling oxidants or other important metabolic oxidation–reduction reactions. However, it can reduce highly reactive oxidants such as the hydroxyl radical and pernicious peroxynitrite molecule [49]. However, the radical scavenging effect of molecular hydrogen cannot explain all its pleiotropic effects. It also influences signal transduction, modulates miRNA expression, and regulates protein phosphorylation cascades, resulting in diverse biological and cellular effects [36]. Molecular hydrogen has also been shown to induce the expression of PGC-1α and exert antioxidant effects due to various proposed mechanisms [50]. One of the main ways that H₂ exerts its antioxidant effect is, as mentioned previously, via the induction of the Nrf-2/keap1 pathway and the subsequent expression of various antioxidant enzymes including induction of heme-1 oxygenase [20]. Furthermore, in addition to its ability to increase PGC-1α levels and promote mitochondrial biogenesis, H₂ also induces the mitochondrial unfolded protein response (mtUPR) and alleviates mitochondrial stress induced by rotenone, an inhibitor of the mitochondrial electron transport chain complex I [51]. The induction of mtUPR leads to the rejuvenation of compromised mitochondria [52]. Clinical studies have reported
that ingestion of hydrogen water exerted favorable effects on subjects with mitochondrial myopathies, such as an improved lactate/pyruvate ratio and decreased serum matrix metalloproteinase 3 [53].

The previous collective research showing favorable results using PQQ and H₂ on Nrf2 and PGC-1α is corroborated by our in vitro study using Alpha Hope, a novel H₂-producing, PQQ-containing supplement. We found that Alpha Hope dose-dependently increased both PGC-1α and Nrf-2 levels. However, the specific contribution of PQQ and H₂ is presently unknown and may be a focus of future research. It is possible that because PQQ and molecular hydrogen exert their effects via different mechanisms, the PQQ and H₂ combination may promote a greater response, either additively or synergistically. However, it is also possible that there is a threshold response in that once a specific pathway or molecule is activated it cannot be further induced. Nevertheless, there seems to be no rationale for these two molecules in combination either being contraindicated or able to negate each other. Indeed, our research belies this proposition and sets the stage for additional mechanistic and clinical investigation. Similarly, the favorable changes observed in our study should be investigated in a larger well-designed human clinical trial using different populations and ideally various doses of each molecule.

5. Conclusions

In conclusion, the results from our in vitro study demonstrate that acute (48 h) treatment of C2C12 myoblasts with Alpha Hope significantly increases Nrf-2 and PGC-1α levels. However, this short-term treatment did not significantly increase citrate synthase activity. We also demonstrated in a small preliminary human study that four weeks of Alpha Hope supplementation is safe and well tolerated and may favorably influence certain biomolecules within a subset of healthy individuals. However, while this was an exploratory investigation, with a small sample of predominantly women, future studies with a much larger sample of subjects are needed to determine the true impact of Alpha Hope on health-related outcomes in men and women. In accordance with the published literature and our observations, Alpha Hope may represent a novel, simple, and safe supplement for daily self-care by optimizing cell care.

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