

# Ubisol Coenzyme Q10 Promotes Mitochondrial Biogenesis in HT22 Cells Challenged by Glutamate

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## Research article

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## Abstract

**Background:** Glutamate-induced excitotoxicity is a well-recognized cause of neuronal cell death and nutritional supplementation with Coenzyme Q10 (CoQ10) has previously been shown to have neuro-protective actions against it. The objective of this study was to determine whether the protective effect of CoQ10 against glutamate could be attributed to stimulating mitochondrial biogenesis.

**Results:** The mouse hippocampal neuronal HT22 cells were incubated with glutamate with or without ubiquinol Q10 treatment. Significant deterioration of cells after glutamate exposure was observed under a light microscope and cell viability assay, along with a significant drop in clonogenic ability. Glutamate significantly decreased the mitochondrial biogenesis related protein levels of Akt, CREB, PGC-1 $\alpha$ , and NRF2, and reduced mitochondrial biogenesis assessed by a mitochondrial biogenesis kit. Pretreatment with CoQ10 prevented the decreases of Akt, CREB, PGC-1 $\alpha$ , and NRF2 and increased mitochondrial biogenesis.

**Conclusions:** Taken together, these results describe a new mechanism of CoQ10-conveyed neuro-protection and indicate a central role for mitochondrial biogenesis in protecting against glutamate-induced excitotoxicity.

## Background

### Glutamate toxicity and its contribution to neuronal injury

Glutamate excitotoxicity is a condition in which excess glutamate accumulates in the central nervous system (CNS) causing acute neuronal injury and long-term neurodegeneration [1]. Abrupt increases in glutamate are known to accompany traumatic brain injuries, and cerebral ischemia, as well as contribute to neurodegenerative diseases, such as amyotrophic lateral sclerosis, fibromyalgia, multiple sclerosis, and Alzheimer's, Parkinson's, and Huntington's disease. Glutamate-induced injury is primarily the result of increased intracellular calcium levels facilitated by the engagement of glutamate receptors [2]. In addition to Ca<sup>2+</sup> overload, receptor stimulation can lead to collapse of the electrochemical gradient, and activation of protein kinases and endonucleases. These changes accelerate cell death through multiple pathways and through degradation of important substances [3]. Work in our own lab has shown that HT22 mouse hippocampal cells, exposed to glutamate, experience mitochondrial dysfunction causing formation of the mitochondrial permeability transition pore (mPTP). This occurred with increased calcium retention, alteration of the mitochondrial membrane potential (MMP), mitochondrial fragmentation, apoptosis inducing factor (AIF) release, and DNA fragmentation [4, 5].

Much work has been done on how to best limit the effects of glutamate to prevent overstimulation of its receptors. We have found that supplementation with coenzyme Q10 (CoQ10), can prevent many of the glutamate- or rotenone-induced changes within the mitochondria, and improve viability of neurons [4, 6–9]

# CoQ10 and its protective role against mitochondrial damage

One of the principal defenses against mitochondrial dysfunction, is the detoxification of reactive oxygen species (ROS) whose stimulation is both triggered and enhanced when mitochondria are damaged, for example when the MMP is disrupted. CoQ10 is a major cofactor of the electron transport chain (ETC) where the bulk of ROS are produced. Within the ETC, CoQ10 plays a key role in transporting electrons between complexes I, II, and III. It is also recognized as having antioxidant functions. Thus, when ETC function is disrupted by a mitochondrial insult, CoQ10's location positions it as a key antioxidant to reduce oxidative damage [10]. However, continuous, high levels of oxidative stress can lead to CoQ10 depletion and prevent adequate detoxification of ROS.

Therefore, exogenous antioxidant supplementation of CoQ10, can improve outcomes after mitochondrial damage [6–8], including from glutamate exposure [4]. CoQ10 is produced naturally within cells as part of the cholesterol pathway, but the cellular content can be further increased through consumption of food sources high in CoQ10, such as meat, nuts and green leafy vegetables. Exogenous ubiquinone (oxidized form of CoQ10), or ubiquinol (a reduced and more bioavailable form of CoQ10 [11]), can also be administered. This additional supplementation may particularly benefit aging adults as CoQ10 levels naturally decline with age [12].

Benefits from CoQ10 supplementation were noted in a number of clinical trials. For example, in chronic heart failure patients, CoQ10 improved mortality, reduced the incidence of hospital stays, and improved patients' functional classification by the New York Heart Association [13]. CoQ10 increased total antioxidant enzymes and reduced inflammatory biomarkers in diabetic hemodialysis patients [14,15]. CoQ10 benefits were also reported in a clinical trial for fatigue associated with multiple sclerosis [16]. More trials are needed to establish the efficacy of CoQ10 in preventing neurodegeneration, and in preserving mitochondrial function. Our lab has been interested in the neuroprotective benefits of CoQ10 supplementation. We believe this protection can be attributed to the effects of CoQ10 on the mitochondria, beyond antioxidant functions. In this study, our goal was to determine if CoQ10 could increase mitochondrial biogenesis to improve outcome against glutamate toxicity.

## Increasing mitobiogenesis may protect neurons against glutamate-induced damage

Mitochondrial biogenesis is a process by which new mitochondria are formed through the growth and division of pre-existing mitochondria [17]. Activation of this process often occurs during an insult to the cell. This is an attempt to counteract the damage response. Essentially, increasing numbers of mitochondria serves to boost ATP production, and increase detoxification of ROS. Unfortunately, this response is usually not sufficient to counteract the damage response [18]. However, because of its protective effect, enhancing the mitochondrial biogenesis process is a neuroprotective strategy [19].

Changes in mitochondrial biogenesis can be determined by examining expression of key proteins mediating mitochondrial biogenesis pathways. Peroxisome proliferator-activated receptor gamma, coactivator 1 alpha (PGC1- $\alpha$ ) appears to be the master regulator of mitochondrial biogenesis [17]. Once activated, either through phosphorylation or deacetylation, PGC1- $\alpha$  activates the mitochondrial transcription factors, nuclear respiratory factor 1 and 2 (NRF1,2). The NRF 1,2 could activate the mitochondrial NDA and protein synthesis either directly or through activating mitochondrial transcription factor A (TFAM) [20]. Activation of these transcription factors by PGC1- $\alpha$ , leads to the synthesis of mitochondrial genes encoded in nuclear and mitochondrial DNA, promoting generation of new mitochondria.

The overall objectives of this work were to determine if glutamate exposure itself affected mitochondrial biogenesis in hippocampal cells and if supplementation with CoQ10 conferred protection against glutamate-induced toxicity by promoting mitochondrial biogenesis. To this end, we measured mitochondrial biogenesis in hippocampal cell exposed to glutamate, with and without CoQ10 pre-treatment. We further analyzed mitochondrial biogenesis protein expression patterns to determine the molecular mechanisms involved. Given the active role mitochondria play in many disease models, including neuronal injury from glutamate excitotoxicity, we believe elucidating mitochondrial changes, and targeting them for correction, may improve therapeutic outcomes in patients.

## Methods

### Cell Culture

HT22 is a mouse hippocampal cell line kindly provided by Dr. Jun Panee at the University of Hawaii. HT22 cells were cultured as previously described [20]. Stock preparations of glutamate (Sigma-Aldrich, St. Louis, MO) and CoQ10 supplement, Ubisol-Q10 (Zymes LLC, Hasbrouck Heights, NJ), dissolved in water, were diluted with cell culture media before being added to cells. Cell viability assays tested glutamate concentrations from 1–8 mM after 24 h exposure and, where indicated, Ubisol-Q10 (25  $\mu\text{g}/\mu\text{L}$ ) was added 24 h prior to glutamate addition. In all other experiments, a single concentration of glutamate (4 mM) was used.

### Measurement of cell viability via resazurin assay

Cell viability was measured using a water-soluble, indicator dye, resazurin (7-Hydroxy–3H-phenoxazin–3-one 10-oxide) (Acros Organics, NJ) as previously described [21]. Metabolically active cells convert the resazurin sodium salt into a fluorescent compound, resorufin, allowing comparison of the fluorescence generated among experimental groups relative to the control. Thus, fluorescence generated is a measure of viability. Fluorescence was measured using a PHERAstar Microplate Reader (BMG Labtech, NC) with a 540–20/590–20 excitation/emission filter. Cell viability was calculated using the formula: (Fluorescence Intensity of Experimental/Average Fluorescence Intensity of Control)  $\times$  100 = % of viable cells. Statistical significance was measured using two-way ANOVA and Bonferroni's multiple comparisons test.

## Western blotting

After undergoing experimental treatments for 24 h (untreated, glutamate alone (4 mM), CoQ10 alone (25 ug/uL), and glutamate plus CoQ10 24 h pre-treatment), cells were harvested and washed with phosphate-buffered saline (PBS) before lysing to obtain either total protein extracts, or protein fractions from cytosolic, mitochondrial, and nuclear compartments. Total protein extracts, cytosolic, and mitochondrial protein fractions were prepared as previously described [22] and we obtained nuclear protein fractions as described by Andrews and Faller [21].

Protein concentrations were determined through standard Bradford assays (Bio-Rad Laboratories, Hercules, CA). Western blot analysis was performed essentially as previously described [20] separating 20 µg of protein lysate using 4–12 % Bis-Tris NuPAGE gels (Invitrogen, Carlsbad, CA) and the Bio-Rad Mini Trans-Blot system to transfer proteins to Polyvinylidene fluoride membranes. The following primary antibodies from Cell Signaling Technology (Danvers, MA) were used at 1:1000 dilutions: anti-AKT, anti-pAKT, anti-CREB, anti-pCREB, anti-Cox IV, and anti-Histone 3. Anti-β-actin was also used at a 1:5000 dilution. Anti-PGC-1α (1:1000) was obtained from Abcam (Cambridge, UK). Anti-NRF2 (1:200) was from Santa Cruz Biotechnology (Dallas, TX) and anti-TFAM (1:1000) was from Calbiochem (San Diego, CA). IRDye 680LT goat anti-rabbit, IRDye 800CW goat anti-mouse, and donkey anti-goat secondary antibodies from Li-COR, Inc (Lincoln, NE) were used at 1:10,000 dilutions for visualization using the Li-COR Odyssey Classic Imaging System scanner. Scans were analyzed with the Li-COR Image Studio Software version 5.2.5. as previously described [22]. Statistical significance was measured using one-way ANOVA and Bonferroni's multiple comparisons test.

## Clonogenic assay

Clonogenic assays measured the reproductive potential of HT22 cells after exposure to glutamate and rescue by CoQ10 treatment. Cells were treated as described before for untreated, glutamate, CoQ10, and glutamate plus CoQ10 experimental groups. After treatment, cells were harvested and seeded at a density of 100 cells per 10 cm dish, except in the case of the glutamate only group, which were seeded at a higher density of 5000 cells per dish. Cells were incubated under standard culture conditions for 14 days to allow visible colonies to form from individual cells seeded. Colonies were stained with a 0.05 % crystal violet solution. Number of colonies were counted using ImageJ software version 1.49 [23]. Plating efficiencies were calculated by taking the actual number of colonies formed in each plate and dividing by the number of cells originally seeded and then multiplying by 100 to obtain a percent. The percent of cells surviving was calculated by taking the individual plates' plating efficiencies and dividing by the average plating efficiency of the untreated, control group and again multiplying by 100. The average percent survival for each group was quantified and analyzed for statistical significance using one-way ANOVA and Bonferroni's multiple comparisons test.

## Measurement of mitochondrial biogenesis

Different levels of mitochondrial biogenesis among experimental groups were assessed using a MitoBiogenesis™ In-Cell ELISA Kit (Abcam, Cambridge, UK) according to the manufacturer's protocol. Briefly, 20,000 cells per well were seeded in 96 well plates and allowed to adhere overnight. Cells were then fixed with 4 % paraformaldehyde, briefly permeabilized with TritonX-100, blocked, and incubated overnight at 4 °C with primary antibodies. Primary antibodies were specific against mitochondrial DNA-encoded protein, COX-I, and nuclear DNA-encoded protein, SDH-A. Cells were washed and incubated for 1 h at room temperature with a solution of secondary antibodies containing an AP-labeled antibody specific for SDH-A and an HRP-labeled antibody specific for COX-I. The reactions were sequentially developed; first with an AP development solution, and then an HRP development solution. Fifteen minute kinetic reactions with 1 min intervals were recorded using a PHERAstar microplate reader (BMG Labtech, NC) to measure optical density at 405 nm for AP development and 600 nm for HRP development. Whole cell staining with Janus Green was also done to compensate for variations due to cell loss during the procedure. The AP and HRP data were normalized to the Janus Green optical density at 595 nm. The signal ratio of COX-I/SDH-A was calculated to determine increased production of mitochondrial DNA-encoded protein without a matching increase in nuclear DNA-encoded protein. Such an increase was interpreted as an increase in mitochondrial biogenesis. Statistical significance was measured using one-way ANOVA and Bonferroni's multiple comparisons test.

## Statistical analysis

Each experiment was repeated at least three times. Data is presented as either mean values  $\pm$  standard deviation (SD), or as a percentage of the control. Statistical analysis was carried out using ordinary one- or two-way ANOVA with Bonferroni's multiple comparisons test where indicated.  $p$ -values  $\leq 0.05$  were considered statistically significant.

## Results

### CoQ10 protects against cellular damage caused by glutamate exposure

Because the toxic effect of glutamate exposure can vary between cell lines, we first looked at glutamate-induced loss of viability in HT22 cells using a range of glutamate concentrations from 1 to 8 mM. Cells were exposed to these concentrations for 24 hours before adding the resazurin dye as described in the materials and method. Figure 1A shows a dose-dependent decrease in cell viability as glutamate concentration rises. At 4 mM, glutamate decreased viability by roughly 40 % ( $p < 0.001$ ) and at our highest concentration of 8 mM, more than 80 % of cells were affected. Cells that were treated for 24 h with CoQ10 (25  $\mu\text{g}/\mu\text{L}$ ) before the addition of glutamate, did not experience these losses in viability, even at the highest doses of glutamate tested. Even though CoQ10 conferred significant protection from these higher concentrations of glutamate, we decided to use 4 mM glutamate in our subsequent experiments as this dose delivered an appropriate insult without killing too many cells.

As can be seen in Figure 1B, 4 mM glutamate caused significant morphological changes corresponding to reduced proliferation and induction of cell death. Microphotographs taken at 10X magnification under a light microscope showed glutamate-exposed cells rounding up and detaching from the culture flask surface. There was marked reduction in cell proliferation compared to untreated cells. CoQ10 treatment alone did not appear to affect cell proliferation or induce cell death. CoQ10 pretreatment, however, effectively prevented the morphological changes caused by glutamate.

## **Clonogenic ability, inhibited by glutamate, is retained when cells are pre-treated with CoQ10**

In addition to reducing cell viability directly, cells surviving glutamate exposure may lose their proliferative capability even after glutamate has been removed. We used clonogenic colony forming assays to measure the ability of single cells to form colonies, an indication that they have retained their reproductive ability and are still capable of cellular division. Our experiments assessed this ability in cells that survived the initial 24 h glutamate exposure, CoQ10 treatment alone, or glutamate plus CoQ10 pretreatment. Surviving cells were harvested, washed and then seeded at low densities to allow individual cells to form visible colonies.

After staining colonies with crystal violet, the plating efficiency (PE) for each group was calculated by taking the number of colonies formed and dividing it by the number of cells seeded and then multiplying by 100 to obtain a percent. We found that in untreated cells, the average PE was 68 %. Our results showed the PE was reduced in glutamate-exposed cells dropping to 13 % but was not significantly altered from control in the CoQ10 alone, or the glutamate plus CoQ10 group. To find the surviving fraction (% survival graphed in Figure 1C) we divided the PE of the treated sample by the PE of the control cells and multiplied by 100. Doing this set our untreated control group at 100 % survival and our experimental groups were reported relative to this value. Glutamate exposure dropped survival to 19 % ( $p < 0.001$ ). CoQ10 alone and CoQ10/glutamate groups were not significantly different from untreated cells with average survival fractions of 93 and 101 % respectively.

## **Glutamate exposure decreases mitochondrial biogenesis while CoQ10 promotes it**

Labeling both a mitochondrial DNA-encoded protein (COX-I) and a nuclear DNA-encoded protein (SDH-A), we measured increases, or decreases, in COX-1 expression over SDH-A that were interpreted as corresponding to increases, and decreases, in mitochondrial biogenesis, respectively. AP and HRP enzyme-catalyzed reactions (as described in methods) were developed for 15 min with optical density measured at 1 min intervals. Figure 2A shows the ratio of the COX-1 signal to the SDH-A signal (normalized to Janus Green) at each interval reading. The bar graph (Figure 2B) shows the average ratio for each experimental group at the 15 min endpoint. We observed that the COX-1/SDH-A ratio from glutamate exposure was depressed indicating reduced mitochondrial biogenesis, but did not meet the

threshold for statistical significance. Compared to control, CoQ10 treatment alone significantly increased mitochondrial biogenesis by roughly 60 % ( $p < 0.01$ ). When glutamate was added, after pretreatment with CoQ10, there was still a CoQ10-driven increase in mitochondrial biogenesis, but it was not enough to meet statistical significance.

## **Glutamate and CoQ10 affect the master regulator of mitochondrial biogenesis, PGC-1 $\alpha$**

PGC-1 $\alpha$  is considered the master regulator of mitochondrial biogenesis through its action as a transcription factor for genes central in promoting mitochondria growth. We show in Figure 3 that glutamate exposure significantly reduced PGC-1 $\alpha$  expression by 40 % in HT22 cells after 24 hours ( $p < 0.01$ ). Treatment with CoQ10 alone had no effect on PGC-1 $\alpha$  expression, but when added prior to glutamate addition, it significantly improved PGC-1 $\alpha$  expression increasing it by 35 % from glutamate exposure alone ( $p < 0.05$ ).

PGC1- $\alpha$  is most commonly activated by either phosphorylation, or deacetylation, of its promoter region. A number of endogenous and exogenous actors can facilitate these reactions. A noted inducer of PGC1- $\alpha$  phosphorylation is the AKT/CREB pathway [16], therefore, we measured expression of AKT and CREB in our experimental groups. Although, phosphorylated AKT (pAKT) and phosphorylated CREB (pCREB) were not significantly reduced from glutamate exposure, they were both significantly upregulated when CoQ10 was added (Figure 4;  $p < 0.05$ ).

## **Regulation of downstream PGC-1 $\alpha$ effectors are also altered during glutamate exposure and restored by CoQ10 treatment**

The downstream effectors, NRF2 and TFAM, were also measured (Figure 5). We observed a nearly 70 % decrease in NRF2 expression after glutamate exposure ( $p < 0.001$ ). Addition of CoQ10 resulted in complete restoration of NRF2 from this glutamate-inhibited level ( $p < 0.001$ ). Finally, TFAM expression was measured in the mitochondrial compartment. Unlike NRF2, TFAM was not reduced by glutamate exposure, however, there was a slight, but significantly increase in TFAM expression (20 %) from control levels when CoQ10 was added ( $p < 0.05$ ).

## **Discussion**

Damage from glutamate excitotoxicity is exacerbated by oxidative stress [5] and increasing the number of mitochondria is a way to boost the antioxidant, detoxification functions of cells against this stress. A study by Wang et al., showed that NAD<sup>+</sup> was able to protect against glutamate-induced apoptosis through increasing mitochondrial biogenesis [23]. We believe CoQ10 similarly protects against cell death by boosting mitochondrial biogenesis (Figure 2). Protecting PGC-1 $\alpha$  expression may be crucial to this effect. CoQ10 treatment provided restoration of glutamate-diminished PGC-1 $\alpha$  protein expression (Figure

3), while at the same time increasing cell viability and clonogenic potential (Figure 1). We expanded our understanding of this mechanism by exploring alterations to PGC-1 $\alpha$  signaling pathways both upstream and downstream this central regulator.

Upstream of PGC-1 $\alpha$ , we observed that both AKT and CREB phosphorylation were increased with CoQ10 pretreatment, after being reduced by glutamate exposure (Figure 4). It is interesting to note that, while PGC-1 $\alpha$  was significantly reduced with glutamate exposure, there was no direct effect on AKT and CREB. This indicates that PGC-1 $\alpha$  was directly inhibited by glutamate, or there was an upstream effector of PGC-1 $\alpha$  (other than AKT and CREB) that we failed to identify. CoQ10, however, did directly boost AKT and CREB activation. This increase may have counteracted any unidentified upstream effectors of PGC-1 $\alpha$  that were reduced by glutamate exposure. More work is needed to determine how glutamate diminishes PGC-1 $\alpha$  expression. Regardless, our work is in line with other reports. For example, PGC-1 $\alpha$  and NRF1 protein expression were all reported to be significantly reduced by glutamate in primary cultured cortical neurons [23].

While we did not observe a change in NRF1 in our model, we did observe significant degradation of NRF2 from glutamate exposure. NRF2 is a transcription factor, activated under stress conditions, to increase survival through enhancing transcription of genes involved in anti-inflammatory, antioxidant, and general cytoprotective pathways [24]. Therefore, seeing a decrease in NRF2 expression with glutamate exposure (Figure 5) was not surprising. Stimulated by PGC-1 $\alpha$ , NRF2 has been shown to be a key contributor to mitochondrial biogenesis pathways [25, 26]. NRF2's upregulation after CoQ10 treatment (Figure 5) may thus contribute to our observed increase in mitochondrial biogenesis (Figure 2). This effect could be achieved either directly activating mitochondria DNA and protein synthesis or indirectly through activating NRF2 [27, 28].

TFAM is an important mitochondrial transcription factor integral to maintaining the mitochondrial genome by regulating its packaging, stability, and replication [29]. While we did not observe a significant decrease in TFAM after glutamate exposure, it was significantly elevated by CoQ10 treatment (Figure 5). This would indicate that in the TFAM is not entirely regulated by NRF2 in our model, since they did not increase and decrease together. Regardless of this pathway dissonance, both NRF2 and TFAM transcription factors play significant roles in mitochondrial health. Our results indicate that NRF2 degradation plays a role in glutamate-mediated toxicity, while TFAM does not. We also showed that NRF2 can be restored with CoQ10 treatment to prevent glutamate-induced toxicity. TFAM can further be upregulated to protect cells against damage and, along with NRF2, promote mitochondrial biogenesis.

## Conclusion

In summary, our results show that glutamate, while causing severe cellular damage, affects mitochondrial biogenesis pathways, particularly through inhibition of PGC-1 $\alpha$  and NRF2, to decrease mitochondrial biogenesis. CoQ10 is an effective option for mitigating this damage. In addition to preventing decreases in PGC-1 $\alpha$  and NRF2, CoQ10 stimulated activity of AKT, CREB, and TFAM. AKT and

CREB may further boost PGC-1 $\alpha$  expression and, along with TFAM, stimulate mitochondrial biogenesis. Glutamate excitotoxicity is involved in the development of neurodegenerative diseases and acute brain injuries, and mitochondrial biogenesis can affect the disease course. Because of this, we believe the mechanisms of CoQ10-mediated mitochondrial biogenesis enhancement should be further explored.

## Declarations

### Abbreviations

Akt, aka Protein kinase B

ATP, adenosine triphosphate

CNS, central nervous system

CREB, cAMP response element-binding protein

CoQ10, coenzyme Q10

COX-I, cyclooxygenase I

ETC, electron transport chain

mPTP, mitochondrial permeability transition pore

NAD, *nicotinamide adenine dinucleotide*

NRF1,2, nuclear respiratory factor 1 and 2

PBS, phosphate-buffered saline

PGC-1 $\alpha$ , peroxisome proliferator-activated receptor gamma, coactivator 1 alpha

ROS, reactive oxygen species

SDH-A, succinate dehydrogenase complex flavoprotein subunit A

TFAM, mitochondrial transcription factor A

### Ethics approval and consent to participate

Not applicable.

### Consent for publication

All authors have reviewed the manuscript and agreed to publish.

## Availability of data and materials

Data are available upon request.

## Competing interests

The authors have declared that no competing interest exists.

## Funding

None.

## Authors' Contributions

Conceived and designed experiments: GC, PAL. Performed the experiments: MZ, MH, QQ, SLM. Wrote the paper: MZ, GC, PAL

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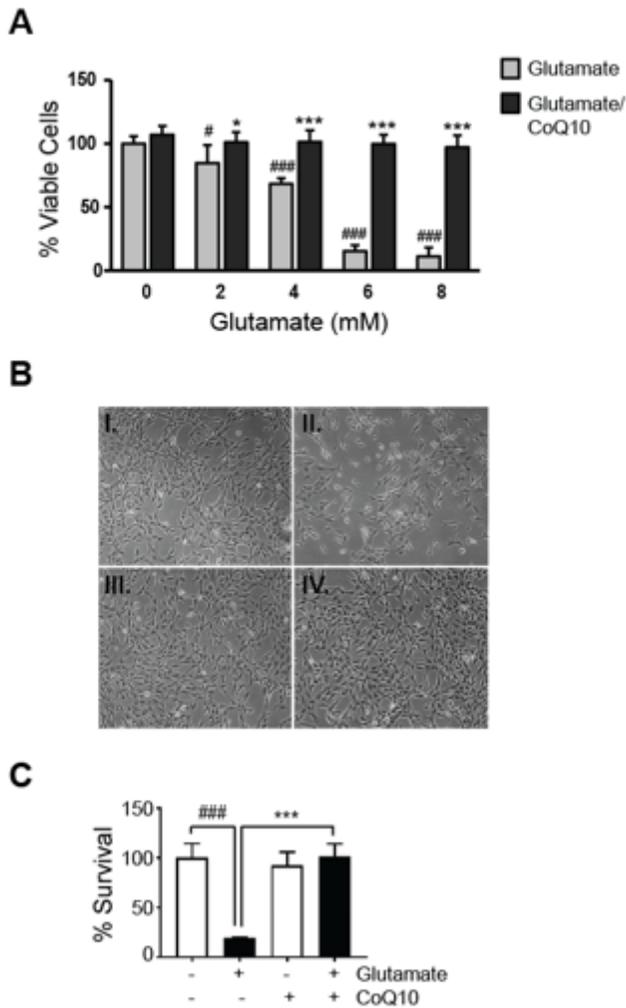
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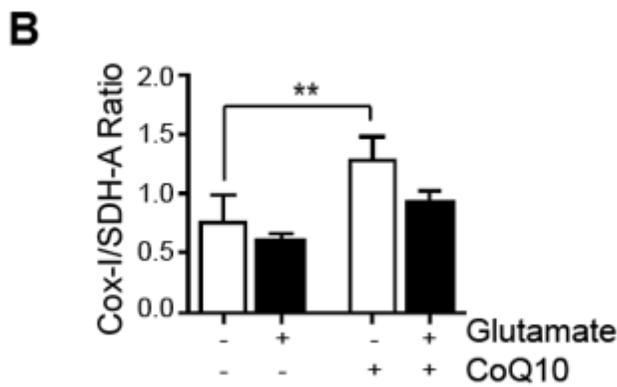
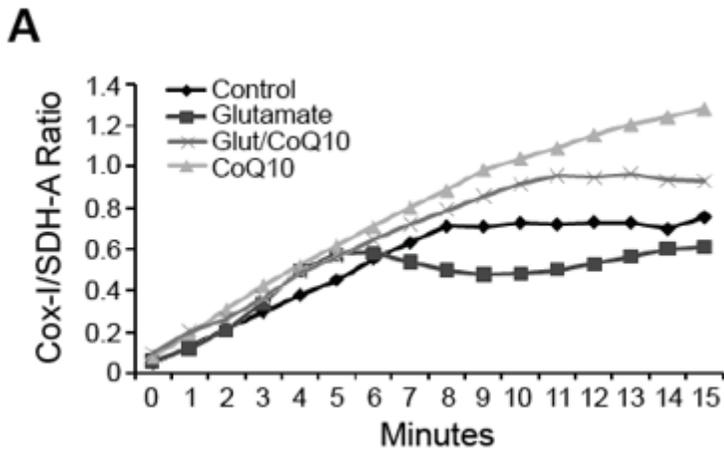
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## Figures



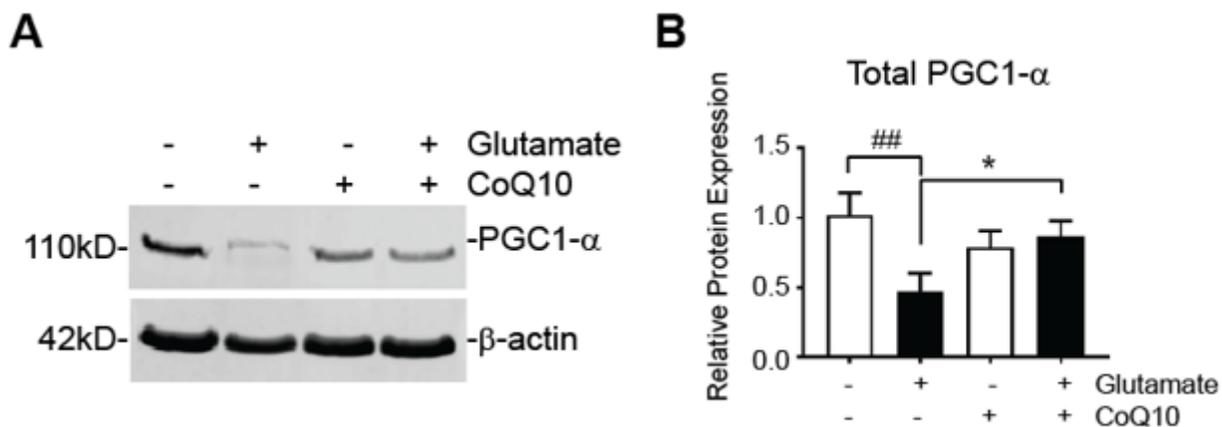
**Figure 1**

CoQ10 prevents losses in cell viability and clonogenic ability during glutamate exposure. A) Glutamate decreases HT22 viability in a dose-dependent manner while pre-treatment with CoQ10 restores it. HT22 cells were treated at 70 % confluence in 96-well plates with the indicated concentrations of glutamate (2-8 mM) with and without CoQ10 (25  $\mu\text{g}/\mu\text{L}$ ) treatment 24 hours prior to glutamate exposure. Cell viability was assessed 24 hours after glutamate addition using the resazurin viability assay. #  $p < 0.05$  vs control, ###  $p < 0.001$  vs control, \*  $p < 0.05$  vs glutamate, \*\*\*  $p < 0.001$  vs glutamate. B) CoQ10 pre-treatment prevents cellular deterioration and reduced growth from glutamate exposure. Cells were treated over the course of 48 hours in the following groups: 1) untreated control, 2) 4 mM glutamate during the last 24 hours, 3) 25  $\mu\text{g}/\mu\text{L}$  CoQ10 for 48 hours, or 4) glutamate was added for 24 hours after pre-treatment with CoQ10 for 24 hours (CoQ10 was not removed during glutamate exposure). After 48 hours, cells were observed under a light microscope at 10X magnification. C) Reduced clonogenic ability occurs after glutamate exposure, but is restored by CoQ10. Cells were treated as in B before harvesting, washing, and seeding in 10 cm dishes as described in the materials and methods to allow colony formation from single cells. Graphed is the average surviving fraction (% survival) in each group. ###  $p < 0.001$  vs control, \*\*\*  $p < 0.001$  vs glutamate.



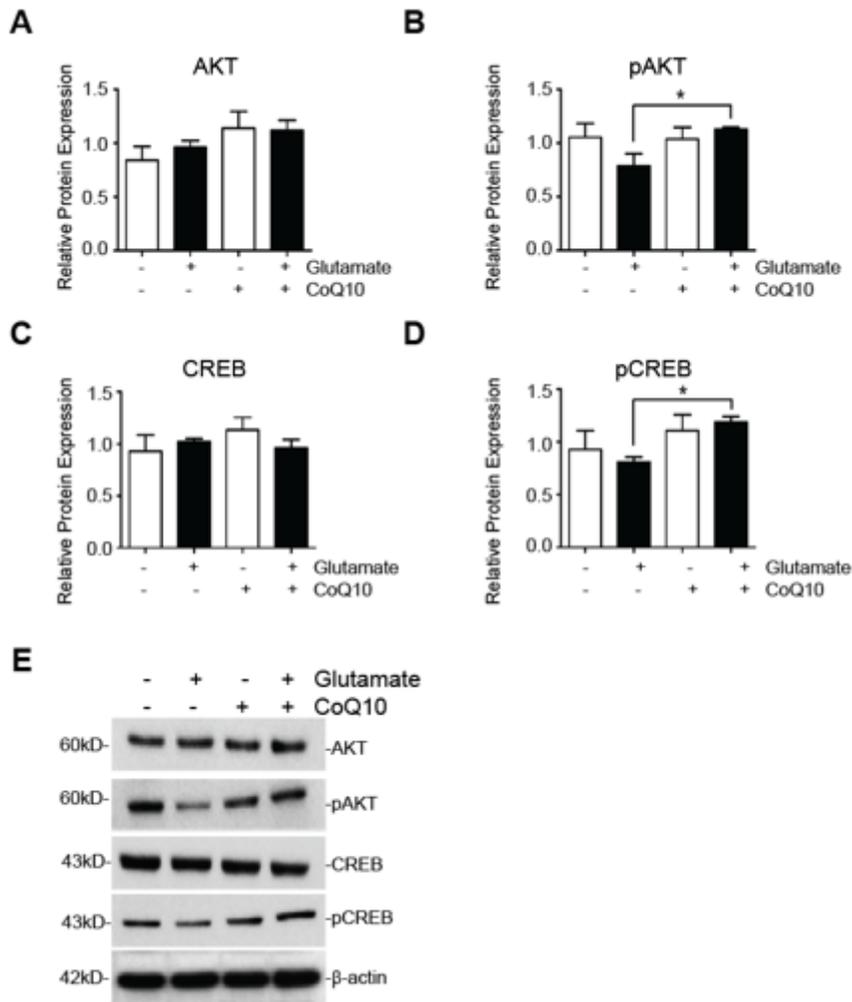
**Figure 2**

CoQ10 causes an increase in mitochondrial biogenesis. A) Cox-I/SDH-A was obtained after treating cells as described in Figure 1B and using an ELISA-based mitochondrial biogenesis detection kit as described in the materials and methods. Cox-1 optical density was detected via HRP enzyme development and SDH-A via AP enzyme development separately over the course of 15 min. The graphed ratios are from the recorded optical densities of each reaction at 1 min intervals and are averages of at least three wells. B) Graphed is the average ratio of mitochondrial DNA-encoded Cox-I over nuclear DNA-encoded SDH-A at the 15 min development endpoint for each treatment group \*\*  $p < 0.01$  vs control.



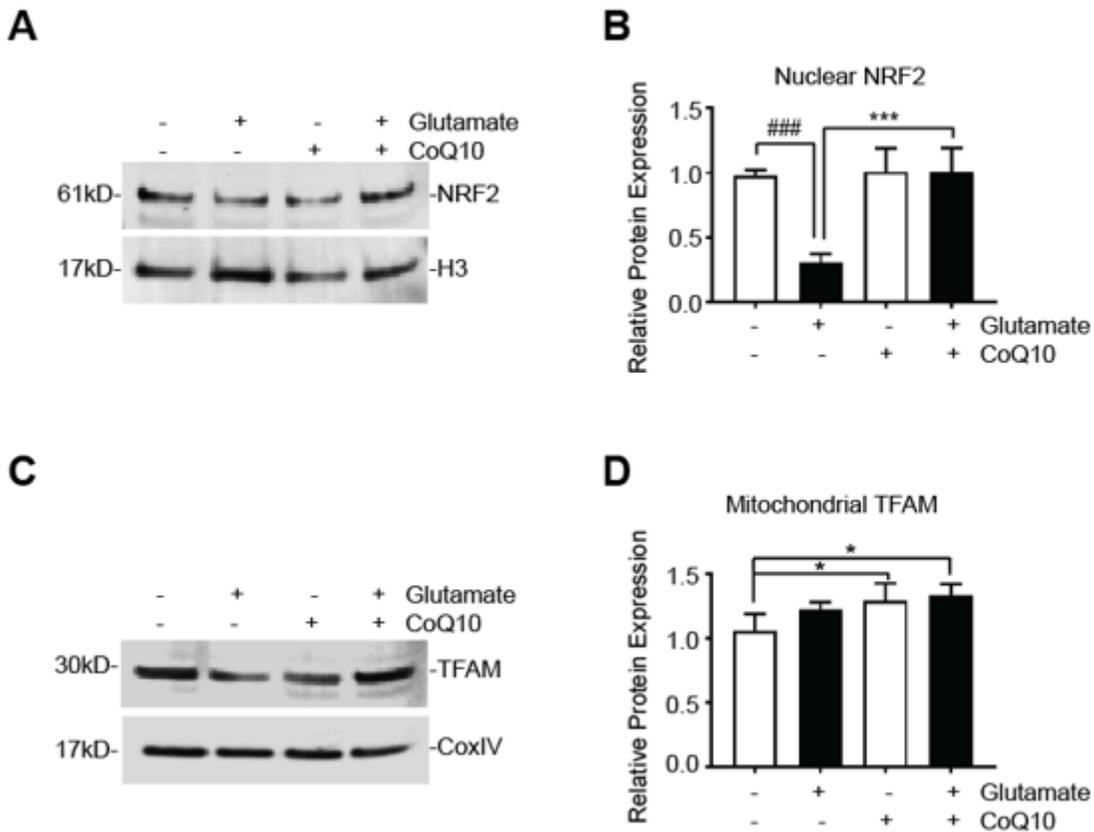
**Figure 3**

CoQ10 pretreatment prevents glutamate-induced reduction in PGC1- $\alpha$  protein expression. A) Representative Western blot image for PGC1- $\alpha$  protein and loading control  $\beta$ -actin. Total protein fractions were obtained from cells treated as described in Fig 1B. B) Western blot analysis showing average protein expression, relative to untreated control, of PGC1- $\alpha$  after normalizing fluorescent signals to  $\beta$ -actin. ##  $p < 0.01$  vs control; \*  $p < 0.05$  vs glutamate. Results are representative of at least three Western blots.



**Figure 4**

Upstream effectors of PGC1- $\alpha$  are modified with glutamate/CoQ10 treatment. Cells were treated as in Fig 1B and lysed for nuclear protein content. Western blot analysis showing average nuclear protein expression, relative to untreated control, of A) AKT, B) pAKT, C) CREB, and D) pCREB after normalizing fluorescent signals to  $\beta$ -actin. Results are representative of at least three Western blots \*  $p < 0.05$  vs glutamate. E) Representative Western blot images for nuclear AKT, pAKT, CREB, pCREB, and loading control  $\beta$ -actin. Samples were treated as in Fig 1B.



**Figure 5**

Downstream PGC1- $\alpha$  effectors, NRF2 and TFAM, are altered with glutamate/CoQ10 treatments. A) Glutamate reduces protein expression of NRF2 which is restored by CoQ10. Cells were treated as in Fig 1B and nuclear protein fractions were analyzed with Western blot. Shown is a representative Western blot image of NRF2 and a nuclear loading control, H3. B) Average nuclear protein expression of NRF2 relative to untreated control. Protein content was normalized to H3 fluorescent signal and results are from at least three separate experiments. ###  $p < 0.001$  vs control; \*\*\*  $p < 0.001$  vs glutamate C) CoQ10 increases expression of TFAM. Cells were treated as in Fig 1B. Mitochondrial protein fractions were obtained and analyzed by Western blot. Shown is a representative Western blot image of TFAM and a mitochondrial loading control, COX IV. D) Average mitochondrial protein expression of TFAM relative to untreated control. Protein content was normalized to COX IV fluorescent signal and results are from at least three separate experiments. \*  $p < 0.05$  vs control.