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Pyrroloquinoline Quinone Stimulates Mitochondrial Biogenesis through cAMP Response Element-binding Protein Phosphorylation and Increased PGC-1α Expression*[S]

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Bioactive compounds reported to stimulate mitochondrial biogenesis are linked to many health benefits such increased longevity, improved energy utilization, and protection from reactive oxygen species. Previously studies have shown that mice and rats fed diets lacking in pyrroloquinoline quinone (PQQ) have reduced mitochondrial content. Therefore, we hypothesized that PQQ can induce mitochondrial biogenesis in mouse hepatocytes. Exposure of mouse Hepa1–6 cells to 10–30 μM PQQ for 24–48 h resulted in increased citrate synthase and cytochrome *c* oxidase activity, Mitotracker staining, mitochondrial DNA content, and cellular oxygen respiration. The induction of this process occurred through the activation of cAMP response element-binding protein (CREB) and peroxisome proliferator-activated receptor-γ coactivator-1α (PGC-1α), a pathway known to regulate mitochondrial biogenesis. PQQ exposure stimulated phosphorylation of CREB at serine 133, activated the promoter of PGC-1α, and increased PGC-1α mRNA and protein expression. PQQ did not stimulate mitochondrial biogenesis after small interfering RNA-mediated reduction in either PGC-1α or CREB expression. Consistent with activation of the PGC-1α pathway, PQQ increased nuclear respiratory factor activation (NRF-1 and NRF-2) and Tfam, TFB1M, and TFB2M mRNA expression. Moreover, PQQ protected cells from mitochondrial inhibition by rotenone, 3-nitropropionic acid, antimycin A, and sodium azide. The ability of PQQ to stimulate mitochondrial biogenesis accounts in part for action of this compound and suggests that PQQ may be beneficial in diseases associated with mitochondrial dysfunction.

Bioactive compounds, such as pyrroloquinoline quinone (PQQ),[2] resveratrol, genistein, hydroxy-tyrosol, and quercetin have been reported to improve mitochondrial respiratory control or stimulate mitochondrial biogenesis (1–5), which is potentially important to a number of health-related issues ranging from increased longevity, improved energy utilization, and protection from reactive oxygen species (6–8). Furthermore, mitochondrial DNA depletion and mutations are associated with cardiomyopathy, lactic acidosis, developmental delay, failure to thrive, and impaired neurological function (9). The response to most biofactors is observed after pharmacological intervention or dietary supplementation, although often neargram amounts per kg of diet, or millimolar quantities, are needed for such responses *in vivo*. PQQ stimulates mitochondriogenesis with the addition of only milligram quantities of PQQ per kg of diet, or micromolar concentrations, *in vivo*. For example, PQQ deprivation depresses mitochondrial function, which is reversed when as little as 200–300 μg of PQQ/kg of diet are added (1, 10). PQQ also remains detectable in tissues when there is no or little dietary exposure (11), which has not been observed for other dietary polyphenolic compounds known to promote mitochondriogenesis.

Recently, PQQ produced by rhizobacterium has been identified as an important plant growth factor (12) and is a possible source of PQQ in plant-derived food. In this regard, the ubiquitous presence of PQQ in a broad range of plants leads to a relatively constant exposure in animal diets. More importantly, levels of PQQ from dietary intake from plants are sufficient to maintain the concentration of PQQ typical of tissues (13). From a chemical perspective, assays that measure redox cycling indicate that PQQ is also 100–1000 times more efficient than other quinones and enediols, such as ascorbic acid (14). PQQ can undergo thousands of reductive or oxidative cycles without degradation or polymerization (14).

Peroxisome proliferator-activated receptor-γ coactivator-1α (PGC-1α) is a transcriptional coactivator that induces mitochondrial biogenesis by binding to nuclear respiratory factors and enhancing their activity (15). Nuclear respiratory factors are transcription factors that bind to cis-acting response elements in the promoter regions of many genes that control mitochondrial gene transcription and mitochondrial DNA replication (15). PGC-1α is also associated with a reduction in reactive

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[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Tables 1 and 2 and Figs. 1 and 2.

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[2] The abbreviations used are: PQQ, pyrroloquinoline quinone; CREB, cAMP response element (CRE)-binding protein; PGC-1α, peroxisome proliferator-activated receptor-γ coactivator-1α; siRNA, small interfering RNA; ROS, reactive oxygen species; IPQ, imidazopyrroloquinoline; FCCP, carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone; PBS, phosphate buffer saline; HBSS, Hanks' balanced salt solution; DMEM, Dulbecco's minimal essential medium; NRF, nuclear respiratory factor; ANOVA, analysis of variance; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

oxygen species (ROS) (16, 17) and protection against various mitochondrial toxins (16). Moreover, the phosphorylation of CREB is known to be an important regulator of PGC-1α (18).

The function of PQQ in mammalian physiology remains controversial. PQQ has been proposed as a vitamin (19), but it has not been demonstrated that PQQ serves as an enzyme cofactor in mammalian tissues (20, 21). Upon appreciation that mitochondrial content can be influenced by PQQ nutritional status and that reported beneficial effects of PQQ may be directly related to mitochondrial function, we hypothesized that PQQ may induce mitochondrial biogenesis through a mitochondrial-related cell signaling mechanism. Given that many mitochondrial-related events are regulated by PGC-1α and nuclear respiratory factors (15), we hypothesized that PQQ may interact with a PGC-1α-related pathway. We used the mouse Hepa1–6 hepatocyte cell line as a model to investigate these hypotheses. We also explored whether PQQ may protect against the toxic effects of mitochondrial electron transport chain inhibition.

EXPERIMENTAL PROCEDURES

Reagents—Mitsubishi Gas and Chemical provided PQQ as a gift. Imidazopyrroloquinoline (IPQ) was produced by incubating PQQ with glycine as described previously (22) followed by separation using a PD-10 desalting column (Amersham Biosciences) and lyophilization. Bovine catalase and bovine copper/zinc superoxide dismutase were purchased from Worthington Biochemical and Sigma, respectively. The mitochondrial toxins (rotenone, 3-nitropropionic acid, antimycin A, and sodium azide) that inhibit separate portions of the electron transport chain (Complexes I-IV) and carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (FCCP), used to uncouple oxidative phosphorylation, were obtained from Sigma.

Cell Culture—Hepa1–6 cells were obtained from American Type Culture Collection (Manassas, VA). Basal culturing medium contained Dulbecco's minimal essential medium (DMEM) with 4.5 g of D-glucose/liter media (Invitrogen) supplemented with 10% fetal bovine serum and 100 units/liter penicillin and streptomycin (Invitrogen).

NRF-1, NRF-2, and CREB Reporter Plasmids—The NRF-1 reporter plasmid (Panomics, Fremont, CA) contains four tandem repeats of the NRF-1 response element (5′- TGCGCACGCGCG-3′) within the pTranslucent plasmid, which contains a thymidine kinase minimal promoter and luciferase reporter gene downstream of the NRF-1 response element. The NRF-2 and CREB reporter plasmids were created by first digesting the NRF-1 reporter plasmid with KpnI and NheI to excise the insert. The insert was replaced by ligating annealed oligonucleotides (see supplemental Table 1; the response element in the sense strand is underlined) containing three tandem repeats of the NRF-2 response element from the mouse Tfam promoter (23, 24) or four tandem repeats of the cAMP response element present in the PGC-1α promoter (18) to the modified reporter plasmid. The pTranslucent plasmid lacking any enhancer sequence was used as a control plasmid (Panomics).

PGC-1α Promoter Reporter Plasmids—The PGC-1α promoter reporter plasmids (18), containing a 2.6-kb portion of 5′-flanking sequence of mouse PGC-1α gene between −2533 and +78 relative to the transcriptional start site, with and without an intact CRE site was provided as a generous gift from Dr. Bruce Spiegalman through the Addgene plasmid depository (Addgene plasmids 8887 and 8888). A plasmid lacking the promoter region (pGL3-Basic, Promega) was used as a control.

Reporter Assays—Cells (1×10^5 cells/well) were plated in 12-well plates and transfected the next day with 0.8 μg of reporter plasmid DNA (NRF-1, NRF-2, CREB, or PGC-1α) and 0.8 μg of β-galactosidase expression vector (pCMVβ, Clontech) per well using Lipofectamine 2000 (Invitrogen). Cell media containing DMEM plus fetal bovine serum were replaced in all wells 5 h post-transfection. Cells were then incubated for 24 h with phenol red-free cell media containing either unsupplemented media (control) or media containing 15 or 30 μM PQQ 12 h post-transfection. Cells were washed with phosphate buffer saline (PBS), and cells were lysed with reporter lysis buffer (Promega) and a single freeze-thaw cycle. Luciferase activity was determined using the Luciferase assay system (Promega) and analyzed using a luminometer (Turner 20/20, Turner Biosystems). β-Galactosidase activity was determined using the β-galactosidase enzyme assay system (Promega) and analyzed using a spectrophotometric microplate reader (PerkinElmer Life Sciences HTS 7000 Plus or Labsystems Multiskan Ascent). Response element or promoter activation was determined as a ratio of luciferase activity to β-galactosidase activity.

PGC-1α Protein Expression by Immunoblotting—Cells (3×10^6 cells/plate) were plated into 10-cm plates. Twenty-four hours later cells were incubated with either control DMEM or media supplemented with PQQ for 24 h. Cells were washed with ice-cold PBS and scraped into ice-cold lysis buffer (20 mM HEPES, pH 7.4, 1 mM EDTA. 250 mM sucrose) containing protease inhibitors (Sigma) and sonicated twice for 10 s on ice. SDS was added after sonication to a final concentration of 0.025%, and protein concentration in the resulting cell lysate was determined by Bradford assay. The resulting protein lysate was resuspended in SDS sample buffer containing β-mercaptoethanol. Total cell lysate (10 μg/well) was separated by SDS-PAGE (Bio-Rad Ready Gels) under reducing conditions and transferred to nitrocellulose membrane. Membranes were blocked overnight at 4 °C in 5% nonfat milk in PBST (PBS containing 0.1% Tween 20) and incubated with PBST with rabbit anti-PGC-1α (0.2 μg/ml; Santa Cruz) for 1 h followed by donkey anti-rabbit IgG conjugated to horseradish peroxidase (1:20,000 in PBST; Amersham Biosciences) for 1 h or mouse anti-β-actin conjugated to horseradish peroxidase (3.3 ng/ml; Santa Cruz) antibodies for 1 h at room temperature. Bands were visualized by chemiluminescence (SuperSignal Femto, Pierce; Western blotting Luminol reagent, Santa Cruz) followed by autoradiography and quantified by densitometry (Quantity One, Bio-Rad).

CREB Phosphorylation by Immunoblotting—Cell protein was isolated as described above but in the presence of both phosphatase inhibitors (Phosphatase inhibitor mixture P2850, Sigma) and protease inhibitors (Protease inhibitor mixture P2714, Sigma). CREB phosphorylation at serine 133 and total

CREB protein were detected by immunoblotting using phosphoserine-133-CREB and total CREB antibodies, respectively (Cell Signaling), as described above, except that Tris-buffered saline replaced phosphate-buffered saline, and bovine serum albumin (Fraction V, Sigma) replaced nonfat milk. The amount of phosphorylated CREB was determined by the amount of CREB detected by the phosphoserine-specific antibody and quantified by densitometry divided by the amount of CREB detected by the total CREB antibody.

Cytochrome c Oxidase Assay—Cells (1×10^6 cells/plate) were plated in 10-cm plates. Twenty-four hours later cells were incubated in PQQ supplemented media for 48 h, and cell protein was isolated as described above, except that cells were homogenized with 12 strokes of a Dounce homogenizer, and SDS was omitted. Cytochrome *c* oxidase activity was assessed as described previously (10).

Citrate Synthase Assay—Cells (5×10^5 cells/well) were plated in 24-well plates. Twenty-four hours later cells were incubated in PQQ-supplemented media for 24 or 48 h, and cell protein was isolated using CelLytic M cell lysis reagent (Sigma) and frozen at −80 °C until assayed. Citrate synthase activity in 10 μg of cell protein (measured by Bradford assay) was assessed using the citrate synthase assay kit (Sigma) according to manufacturer instructions, and changes in absorbance were measured using a microplate reader. Activity was expressed relative to control condition.

Mitotracker Assay—Mitochondria were detected by Mitotracker Green FM staining (Invitrogen). Cells were plated in 96-well plates (1×10^4 cells/well) for the microplate assay or 6-well plates (2×10^5 cells/well) for flow cytometry. After 24 h cells were incubated with PQQ-supplemented media (or control) for 24 or 48 h (only 48 h for flow cytometry). Cells were washed with serum-free DMEM and stained with 100 nM (for flow cytometry) or 200 nM (for the microplate assay) Mitotracker Green FM for 30 min. The unstained control samples were incubated with serum-free DMEM containing no dye but an equivalent concentration of DMSO as the stained sample. After staining, cells were washed three times with serum-free DMEM. For microplate assay, staining was detected on a fluorescence microplate reader (PerkinElmer Life Sciences Wallac Victor2, excitation 485 nm, emission 520 nm), and fluorescence was subtracted from the unstained control and expressed relative to cell number. Stained (and unstained control) cells were analyzed by flow cytometry (BD Biosciences) followed by analysis of median fluorescence intensity of 20,000 events by Cellquest software (BD Biosciences).

Mitochondrial/Nuclear DNA Ratios by Quantitative Real-time PCR—Cells (2×10^5 cells/well) were plated in 6-well plates. Twenty-four hours later cells were incubated in control or PQQ-supplemented media for 48 h. Genomic DNA (containing both mitochondrial and nuclear DNA) was isolated from cells (QIAamp DNA mini kit, Qiagen) according to manufacturer's instructions. Mitochondrial DNA (0.1 ng) and nuclear DNA (10 ng) were amplified by quantitative real-time PCR as described previously (1).

Gene Expression by Quantitative Real-time Reverse Transcription-PCR—Cells (4×10^5 cells/well) were plated in 6-well plates. Twenty-four hours later cells were incubated in control or PQQ-supplemented media for 24 h, and total RNA was isolated from cells using TRIzol (Invitrogen) according to the manufacturer's instructions and diluted to 1 μg/μl in RNase-free water. cDNA was generated from 1 μg of RNA by reverse transcription (Applied Biosystems, Foster City, CA) performed at 48 °C for 30 min followed by 95 °C for 5 min. Primers for mouse PGC-1α, Tfam, TFB1M, TFB2M, and β-actin (see supplemental Table 2) were designed by Primer Express (Applied Biosystems). Relative gene expression was determined by quantitative real-time reverse transcription-PCR as described previously (10) but expressed relative to β-actin.

Oxygen Consumption Assay—Cellular oxygen consumption was measured using the BD Oxygen Biosensor system (BD Biosciences). Cells were plated in 6-well plates at 30% confluency, and after 24 h cells incubated in control media or media supplemented with 30 μM PQQ for 24 h or 48 h. Oxygen consumption was then measured as described below, or cells were then exposed to control media containing 0.004% DMSO or media containing 0.2 μM rotenone (dissolved in DMSO) for 24 h. Cells were dissociated from the plates with trypsin-EDTA (Invitrogen). Cells (5×10^5) were then pipetted into each well. Cells were incubated at 37 °C and 5% CO_2 for 1 h, then changes in oxygen concentration in the media was measured using a fluorescence microplate reader (excitation 485 nm, emission 620 nm, heated to 37 °C). Cells were incubated with 1 μM rotenone immediately after plating or 5 μM FCCP just before reading on the microplate reader. DMSO (0.05% diluted in cell media) was included as a control immediately after plating cells into assay plate because both rotenone and FCCP are dissolved in DMSO. Oxygen consumption was expressed as the difference in relative fluorescence units at 10 and 20 min after the start of the assay, relative to the control condition.

Mitochondrial Superoxide Assay—Cells (1×10^4 cells/well) were plated in 96-well plates and 24 h later incubated in 30 μM PQQ-supplemented or control media for 24 h, then incubated in Hanks' balanced salt solution (HBSS) containing 20 μM rotenone (dissolved in 0.25% DMSO) or control HBSS (containing DMSO) for 1 h at 37 °C. Cells were washed 3 times with HBSS and incubated with 10 μM MitoSOX (Invitrogen) for 30 min at 37 °C. Cells were washed 3 times with HBSS, and mitochondrial superoxide was detected using a fluorescence microplate reader (excitation 485 nm, emission 590 nm, heated to 37 °C).

CREB and PGC-1α siRNA-mediated Gene Knockdown—Cells were transfected with Lipofectamine 2000 and PGC-1α-specific siRNA (sense, 5′-AAGACGGAUUGCCCUCAUUUGtt-3′; antisense, 5′-CAAAUGAGGGCAAUCCGUCUUtt-3′) or control siRNA (sense, 5′-AAGCUUCAUAAGGCGCAUAGCtt-3′; antisense, 5′-GCUAUGCGCCUUAUGAAGCUUtt-3′), as previously designed by Zhang *et al.* (25), or CREB-specific siRNA (sense, 5′-UACAGCUGGCUAACAAUGGtt-3′; antisense, 5′-CCAUUGUUAGCCAGCUGUAtt-3′), as previously designed by Vankoningsloo *et al.* (26), or control scrambled siRNA (sense, 5′-UAGUGCACGCUAAGAAUCGtt-3′; antisense, 5′-CGAUUCUUAGCGUGCACUAtt-3′). This region of the PGC-1α nucleotide sequence is conserved in both mice and rats. Transfection and efficacy of siRNA was confirmed by immunoblotting after transfection of PGC-1α-specific or CREB specific and control siRNA as described above. After transfection (24 h post-transfection), cells were incubated

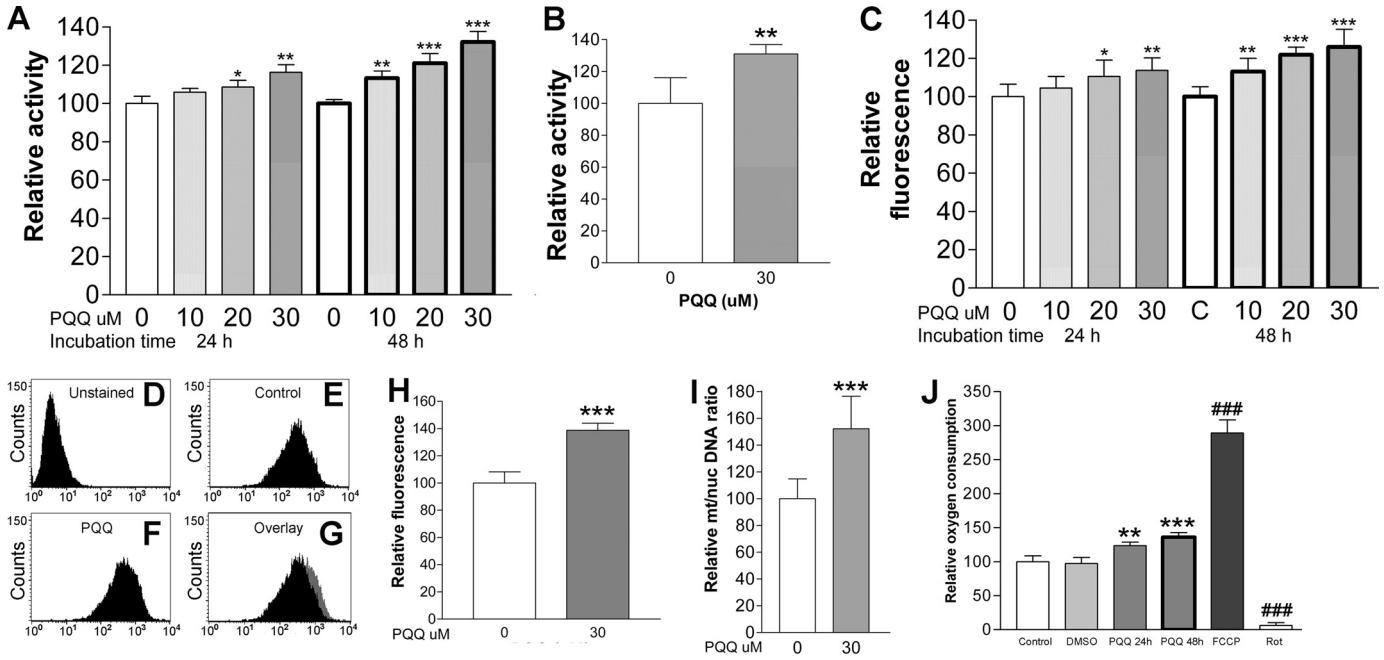

FIGURE 1. **PQQ induces mitochondrial biogenesis.** Increases in cellular mitochondrial content after PQQ exposure were detected by citrate synthase assay (*A*), cytochrome *c* oxidase assay (*B*), Mitotracker staining microplate assay (*C*), Mitotracker flow cytometry assay (*D–H*), mitochondrial to nuclear DNA ratios (*I*), and cellular oxygen consumption (*J*). Citrate synthase activity at 24 or 48 h was determined by MTT reduction assay ($n = 8$) and expressed relative to respective control conditions (without PQQ at 24 or 48 h). Cytochrome *c* oxidase activity at 48 h ($n = 6$) was expressed relative to control condition (without PQQ). A Mitotracker-staining microplate assay after 24 and 48 h ($n = 8$) was expressed relative to respective control conditions (without PQQ at 24 or 48 h). Mitotracker staining flow cytometry assay after 48 h ($n = 5$) was expressed relative to control conditions (without PQQ). Representative histograms (cell counts at each fluorescence intensity, 10^0-10^4, for Mitotracker staining) for unstained (*D*), control (*E*), or 30 μM PQQ medium (*F*) for 48 h are shown, and a control histogram *G*; from *E*, *black*) is overlaid on top of a PQQ histogram (from *F*, *gray*) to show an increase in fluorescence intensity. The *graph* (*H*) represents median fluorescence of Mitotracker staining detected by flow cytometry, expressed relative to control condition (without PQQ). Mitochondrial-to-nuclear DNA ratios were determined by quantitative real-time PCR ($n = 8$) and are expressed relative to control conditions (without PQQ at 48 h). *Columns* and *error bars* indicate the means ± S.D., respectively. *Asterisks* denote significant difference from control condition: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. An oxygen assay ($n = 8$) was determined by increased fluorescence and is expressed as the difference in relative fluorescence units from 10 to 20 min after the assay start relative to control conditions (without PQQ for 24 or 48 h or DMSO 1 h for rotenone (*rot*, contains 0.004% DMSO)). FCCP and rotenone (dissolved in DMSO, added immediately or 1 h before measuring fluorescence, respectively) were used as positive and negative controls, respectively, for oxygen consumption. *Columns* and *error bars* indicate the means ± S.D., respectively. *Asterisks* denote significant difference from control condition (without DMSO) (**, $p < 0.01$; ***, $p < 0.001$), and pound signs indicate significant difference from control condition (with DMSO); ###, $p < 0.001$. Results are representative data from experiments performed at least twice.

with control or PQQ-supplemented media for 48 h, then citrate synthase activity, Mitotracker staining, and mitochondrial DNA content were assessed as described above.

Superoxide and Hydrogen Peroxide Assays—PQQ (2–250 μM for superoxide, 2–50 μM for hydrogen peroxide) was added to control cell media containing 10% fetal bovine serum (without cells) and incubated for 0–24 h (0–6 h for hydrogen peroxide). Superoxide generated by PQQ was measured by luminol oxidation assay (Sigma) on a microplate reader. Hydrogen peroxide generated by PQQ was measured by a xylenol orange-iron oxidation assay (Pierce) on a microplate reader.

Statistics—The effects of PQQ on citrate synthase activity, Mitotracker microplate staining, cellular oxygen consumption, NRF-1, NRF-2, CREB, and PGC-1α promoter activation and cell viability after toxin exposure were analyzed by one-way ANOVA followed by a Tukey post-test. The effects of PQQ on mitochondrial DNA content, cytochrome *c* oxidase activity, Tfam, TFB1M, and TFB2M mRNA expression and PGC-1α mRNA and protein expression were analyzed by Student's *t* test. The effects of PQQ and rotenone on cellular oxygen consumption and superoxide generation were analyzed by one-way ANOVA followed by a Tukey post-test. The effect of PGC-1α and CREB siRNA knockdown on PGC-1α and CREB (for CREB siRNA only) protein expression was analyzed by Student's *t* test. The effects of PQQ after PGC-1α and CREB siRNA knockdown on citrate synthase activity, Mitotracker staining, and mitochondrial DNA content were analyzed by one-way ANOVA followed by a Tukey post-test (and two-way ANOVA where indicated). The effect of PQQ on superoxide and hydrogen peroxide generation (and catalase) in media was analyzed by one-way ANOVA followed by a Tukey post-test. The effect of PQQ and catalase or superoxide dismutase and IPQ on citrate synthase activity, Mitotracker staining, and mitochondrial DNA content was analyzed by one-way ANOVA followed by a Tukey post-test. Significant differences of mean values were established at $p < 0.05$. Unless noted otherwise, statistics reflect intraexperimental variability. The succinate dehydrogenase activity assay is described in the supplemental methods.

RESULTS

PQQ Stimulates Mitochondrial Biogenesis—Citrate synthase activity was increased in Hepa1–6 cells exposed to PQQ for 24 or 48 h (Fig. 1*A*). Cytochrome *c* oxidase activity was increased in cells exposed to PQQ for 48 h (Fig. 1*B*). PQQ treatment increased mitochondrial content, as assessed by Mitotracker microplate assay (Fig. 1*C*) and confirmed by flow cytometry

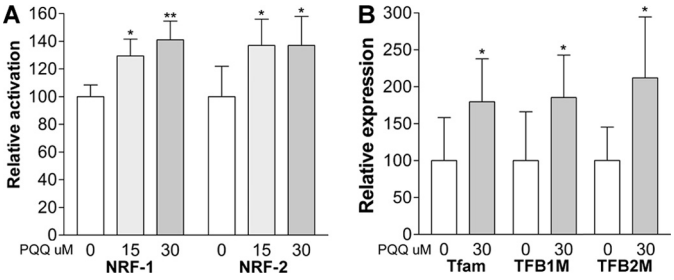

FIGURE 2. **PQQ activates nuclear respiratory factor activity and expression of associated genes.** Cells were incubated in media supplemented with 15 or 30 μM PQQ or control media for 24 h. Activation of NRF-1 and NRF-2 was detected by activation of NRF-1 or NRF-2 response element reporter plasmid (*A*) after PQQ exposure. Activation of Tfam, TFB1M, and TFB2M (*B*), genes that contain response elements for both NRF-1 and NRF-2, was measured by real-time quantitative reverse transcription-PCR. *Columns* and *error bars* indicate the means ± S.D., respectively. *Asterisks* denote significant difference from control condition: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. Results are representative data from experiments performed at least twice.

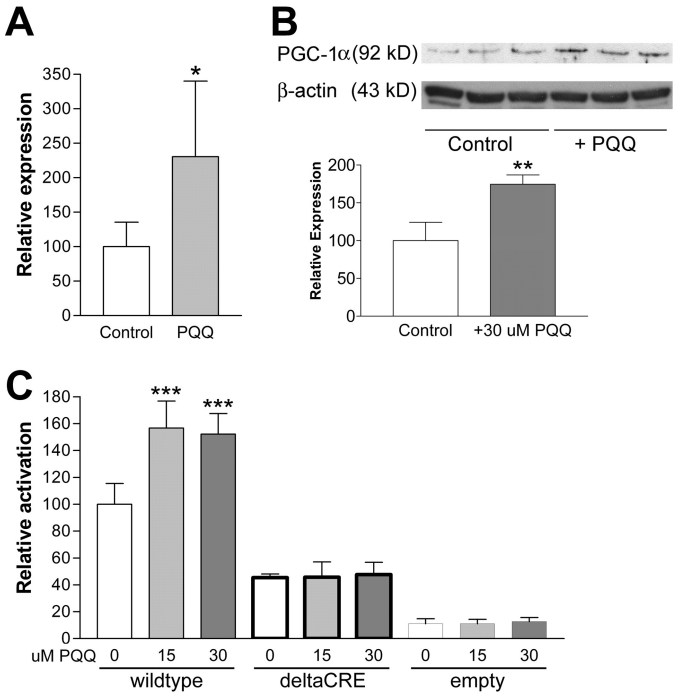

FIGURE 3. **PQQ induces PGC-1α promoter activation and increases PGC-1α mRNA and protein expression.** Cells were incubated in media supplemented with 30 μM PQQ or control media for 24 h. PQQ exposure leads to increased levels of PGC-1α mRNA, detected by quantitative real-time reverse transcription-PCR, and increased PGC-1α protein (*A*), determined by immunoblotting and analyzed by densitometry (*B*). Activation of PGC-1α promoter (*C*), detected by transfection of PGC-1α promoter reporter plasmids, by PQQ requires the presence of a CRE element within the PGC-1α promoter. *Columns* and *error bars* indicate the means ± S.D., respectively. *Asterisks* denote significant difference from control condition, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Results are representative data from experiments performed at least twice.

(Fig. 1, *D–H*). Consistent with an increase in mitochondrial enzyme content, mitochondrial DNA relative to nuclear DNA was also increased in cells after PQQ exposure (Fig. 1*I*). As additional validation, PQQ exposure for 24 or 48 h leads to increased cellular oxygen consumption (Fig. 1*J*).

PQQ Induces Nuclear Respiratory Factor Activation—Because nuclear respiratory factors are known to be key regulators of mitochondrial function (27), we investigated whether PQQ exposure leads to increased activation of these transcription factors. Cells transfected with a reporter plasmid containing response elements for either NRF-1 or NRF-2 displayed increased activation after PQQ exposure (Fig. 2*A*), which suggests that PQQ induces the activation of these transcription factors. PQQ also led to increased Tfam, TFB1M, and TFB2M mRNA expression (Fig. 2*B*). These genes contain response elements for both NRF-1 and NRF-2 (15) and are linked to mitochondrial transcription and replication (27), which suggests that PQQ can activate NRF-1 and NRF-2 activity and related genes and may induce mitochondrial biogenesis.

PQQ Stimulates PGC-1α Promoter Activation and Expression—PGC-1α binding can directly regulate NRF-1 and NRF-2 activity (28). Accordingly, we investigated whether PQQ can regulate PGC-1α. PQQ exposure for 24 h led to an increase in both PGC-1α mRNA (Fig. 3*A*) and protein (Fig. 3*B*). PQQ exposure also led to increased PGC-1α promoter activation but only if a CRE site is retained. No activation of the promoter by PQQ was observed in transfected cells when the CRE site has been mutated (Fig. 3*C*), which suggested that the CRE site in important for PQQ-induced PGC-1α activation, possibly due to CREB (CRE-binding protein).

PGC-1α Is Required for PQQ-mediated Mitochondrial Biogenesis—PGC-1α is required for the induction of mitochondrial biogenesis by PQQ. Transfection with PGC-1α-specific siRNA reduced PGC-1α protein expression (Fig. 4, *A* and *B*) and concomitantly failed to induce mitochondrial biogenesis by PQQ, as measured by citrate synthase activity (Fig. 4*C*), Mitotracker staining (Fig. 4*D*) and mitochondrial DNA content (Fig. 4*E*) compared with the PQQ-induced mitochondrial genesis in cells transfected with the control siRNA. Citrate synthase activity (Fig. 4*C*) and Mitotracker staining (Fig. 4*D*) of cells transfected with PGC-1α siRNA was lower when compared with cells transfected with control siRNA and exposed to control media. Cells transfected with PGC-1α siRNA also showed a trend toward lower mitochondrial DNA content (Fig. 4*E*). Although this result was not statistically significant, significant effects of PGC-1α siRNA and interaction between siRNA and PQQ treatments were observed when analyzed by two-way ANOVA.

PQQ Stimulates the Phosphorylation of CREB at Serine 133—CREB is known to be an important regulator of PGC-1α (18) that facilitates PGC-1α activation (18). Because the CRE site is critical for PGC-1α activation, we determined whether CREB is activated by PQQ. PQQ activates cells transfected with CREB reporter plasmid (Fig. 5*A*), which suggests that CREB is activated by PQQ. Moreover, CREB activity is known to be regulated by phosphorylation (29); therefore, we investigated whether PQQ can induce CREB phosphorylation. PQQ induced CREB phosphorylation at serine 133, which was detectable within minutes of exposure (Fig. 5, *B* and *C*), followed by an eventually decrease in phosphorylation after several hours.

CREB Is Required for PQQ-mediated Mitochondrial Biogenesis—CREB stimulates the expression of PGC-1α expression and is required for the induction of mitochondrial biogenesis by PQQ. Transfection with CREB-specific siRNA reduced both CREB and PGC-1α protein expression (Fig. 5, *D–F*) and

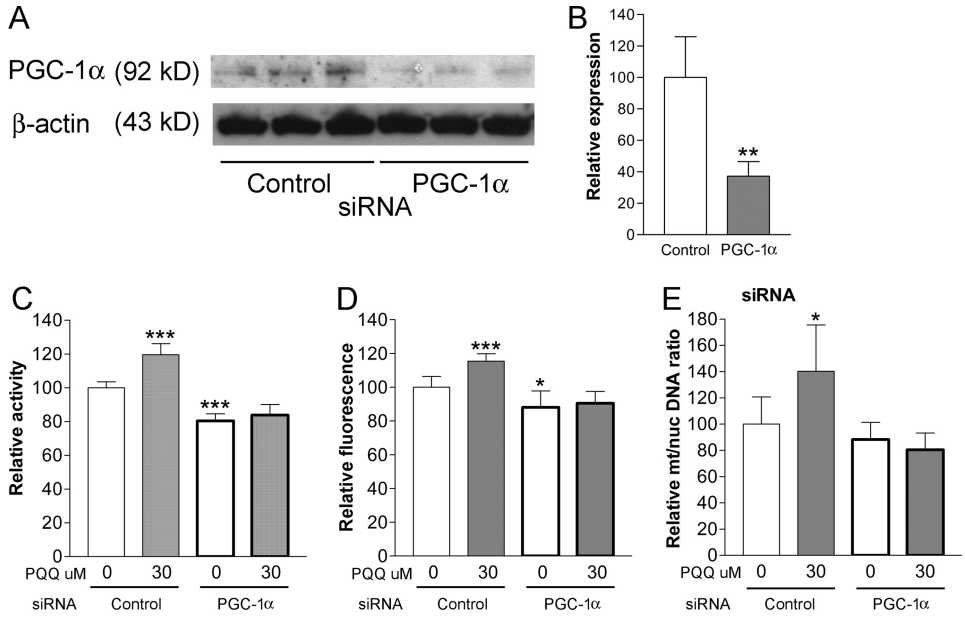

FIGURE 4. **PGC-1α is required for induction of mitochondrial biogenesis by PQQ.** Transfection of PGC-1α-specific siRNA results in reduction of PGC-1α protein expression, detected by immunoblotting (*A*) and analyzed by densitometry ($n = 3$) (*B*). At 24 h post-transfection, cells were incubated in media supplemented with 30 μM PQQ or control media for 24 h. Transfection of PGC-1α-specific siRNA results in loss of PQQ-induced increases in citrate synthase activity, assessed by MTT reduction ($n = 8$) and Mitotracker staining (*C*), assessed by microplate assay ($n = 8$) and mitochondrial DNA content (*D*), and assessed by quantitative real-time PCR ($n = 8$) (*E*). *Columns* and *error bars* indicate the means ± S.D., respectively. *Asterisks* denote significant difference from control condition: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. Results are representative data from experiments performed at least twice.

concomitantly failed to induce mitochondrial biogenesis by PQQ, as measured by citrate synthase activity (Fig. 5*G*), Mitotracker staining (Fig. 5*H*), and mitochondrial DNA content (Fig. 5*I*), compared with the PQQ-induced mitochondrial genesis in cells transfected with the control siRNA. Citrate synthase activity and Mitotracker staining of cells transfected with CREB siRNA and exposed to control media were significantly lower compared with cells transfected with control siRNA and exposed to control media (Fig. 5, *G* and *H*). Cells transfected with CREB siRNA also showed a trend toward lower mitochondrial DNA content (Fig. 5*I*). Although this result was not statistically significant, significant effects of CREB siRNA and interaction between siRNA and PQQ treatments were observed when analyzed by two-way ANOVA.

PQQ-mediated Mitochondrial Biogenesis Is Not Due to Auto-oxidation or IPQ Addition—PQQ has been reported to generate superoxide or hydrogen peroxide in media through auto-oxidation (30, 31). To ensure that the effects of PQQ were not due to this generation of reactive oxygen species, mitochondrial biogenesis and stimulation of NRF-1 and NRF-2 by PQQ were determined in the presence of superoxide dismutase and catalase, similar to methods described by Aizenman *et al.* (32). No differences were observed in the ability of PQQ to stimulate mitochondrial biogenesis or up-regulate NRF-1 and NRF-2 expression when either enzyme was added (Fig. 6, *A–C*). To ensure that superoxide dismutase and catalase were sufficient to metabolize all reactive oxygen species generate by PQQ, superoxide and hydrogen peroxide generated by PQQ was assayed. Similar to data reported by He *et al.* (31), PQQ was found to generate hydrogen peroxide in cell media (Fig. 6*D*). The inclusion of catalase in cell media was sufficient to eliminate hydrogen peroxide generated by PQQ below the level of detection (Fig. 6*E*). No detectable superoxide was generated in cell media by PQQ, as determined by luminol oxidation (data not shown). Because IPQ can be generated from reactions with PQQ and amino acids without bulky side chains (22), cells were incubated with IPQ instead of PQQ. IPQ failed to stimulate mitochondrial biogenesis and did not activate NRF-1 and NRF-2 (Fig. 6, *A–C*), which suggests that the effects observed are due to PQQ, not the PQQ metabolite, IPQ.

PQQ-mediated Induction of Succinate Dehydrogenase Activity Is Dependent on CREB and PGC-1α and Not Due to Auto-oxidation or IPQ Addition—PQQ for 24 and 48 h induced an increase in succinate dehydrogenase activity, as determined by reduction to MTT-formazan (supplemental Fig. 1*A*). Reduction of CREB and PGC-1α expression by siRNA inhibits PQQ-induced increases in MTT reduction (supplemental Fig. 1, *B* and *C*). PQQ stimulated increases in MTT reduction in the presence of superoxide dismutase and catalase. IPQ failed to stimulate an increase in MTT reduction (supplemental Fig. 1*D*). These results are consistent with our other data demonstrating that PQQ induces mitochondrial biogenesis.

PQQ and Cell Viability, Restoration of Oxygen Consumption, and Reduction of Superoxide Formation Associated with Respiratory Inhibition—We investigated whether PQQ can reduce cell death caused by mitochondrial inhibition because of observations that PGC-1α can prevent effects due to mitochondrial inhibition (16, 33). Exposure to rotenone (Fig. 7*A*), 3-nitropropionic acid (Fig. 7*B*), antimycin A (Fig. 7*C*), or sodium azide (Fig. 7*D*) resulted in reduced cell viability compared with cells exposed to media without a mitochondrial toxin. Cells exposed to 10, 20, and 30 μM PQQ supplemented in media for 24 h before exposure to mitochondrial inhibitors displayed increased cell viability compared with corresponding cell cultures without PQQ supplementation (also see supplemental Fig. 2). Moreover, cells exposed to PQQ before rotenone addition also had greater levels of oxygen consumption and reductions in detectable mitochondrial superoxide than cells exposed to rotenone but not PQQ (Fig. 7, *E* and *F*).

DISCUSSION

Previous studies (1, 10) have shown that mitochondrial biogenesis *in vivo* is responsive to dietary PQQ status, but no mechanisms have been established. We show that PQQ influ-

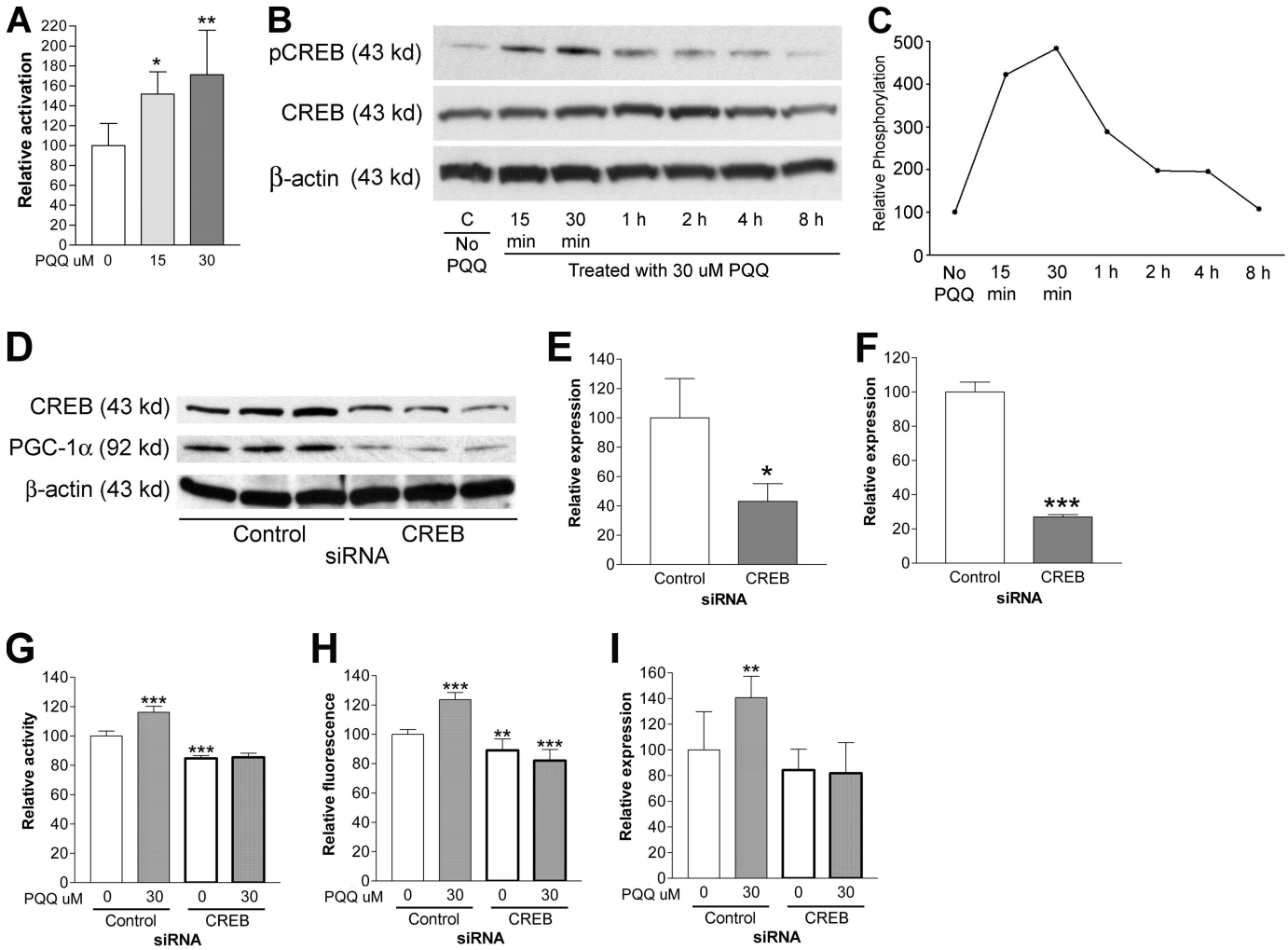

FIGURE 5. **PQQ activates CREB and induces CREB phosphorylation at serine 133, and CREB is required for PQQ-induced mitochondrial biogenesis.** Shown is activation of PGC-1α promoter, detected by transfection of CREB reporter plasmid after PQQ exposure ($n = 6$) (*A*). Phosphorylation of CREB at serine 133 is shown after exposure to 30 μM PQQ (*B*) for times ranging from 15 min to 8 h, detected by immunoblotting using an anti-phosphoserine133-CREB antibody. Phosphorylated CREB was determined by the amount of phosphorylated CREB relative to total CREB (*C*), detected by immunoblotting and determined by densitometry. *Columns* and *error bars* indicate the means ± S.D., respectively. Transfection of CREB-specific siRNA results in a reduction of CREB and PGC-1α protein expression, detected by immunoblotting (*D*) and analyzed by densitometry ($n = 3$) (*E* and *F*). At 24 h post-transfection, cells were incubated in media supplemented with 30 μM PQQ or control media for 24 h. Transfection of CREB-specific siRNA led to a loss of PQQ-induced increases in citrate synthase activity, assessed by MTT reduction ($n = 8$) and Mitotracker staining (*G*), assessed by microplate assay ($n = 8$) and mitochondrial DNA content (*H*), and assessed by quantitative real-time PCR ($n = 8$) (*I*). *Asterisks* denote significant difference from control conditions: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. Results are representative data from experiments performed at least twice.

ences PGC-1α activity, which is a major mechanism for the regulation of mitochondrial biogenesis (15). A part of the process involves CREB, which has been identified as an activator of PGC-1α transcription by binding to the PGC-1α promoter. The induction of PGC-1α by CREB activation is responsive to numerous physiological stimuli, such as catecholamines (34, 35), glucagon (35), and exercise (18). The ability of PQQ to stimulate CREB phosphorylation and activation appears important. PQQ elicited a transient, stimulatory increase in CREB activity. Although the effect lasted less than 8 h, it is similar to the serum stimulation of CREB phosphorylation observed in fibroblasts, which occurs within 1 h of serum exposure and declines after 6 h (36). This response is followed by increases in cytochrome *c* and cytochrome oxidase expression after 12 h (36). The increase in mitochondrial biogenesis and PGC-1α expression was present after 24–48 h after the start of PQQ exposure, which suggests that the increase in PGC-1α activity lasts longer than the increase in CREB activity. One possibility is that the transitory stimulation of PGC-1α expression by CREB leads to a stable increase in PGC-1α activity through a positive autoregulatory feedback pathway. For example, a positive autoregulatory loop for PGC-1 expression for the determination of muscle fiber type from type II to type I involves the binding of MEF2 to the PGC-1α promoter, which is enhanced with MEF2 and PGC-1α coactivation (18). Another positive autoregulatory loop involves the binding of peroxisome proliferator-activated receptor -γ, a co-activation target of PGC-1α, to the distal region of the PGC-1α promoter (37). Thus, it is possible that the activation of PGC-1α by PQQ can induce further lasting increases in either PGC-1α activity or expression by influencing an autoregulatory feedback pathway. Reduction of CREB expression by siRNA-mediated knockdown resulted in a reduction in both CREB and PGC-1α expression. This observation is similar to the reduction in PGC-1α mRNA

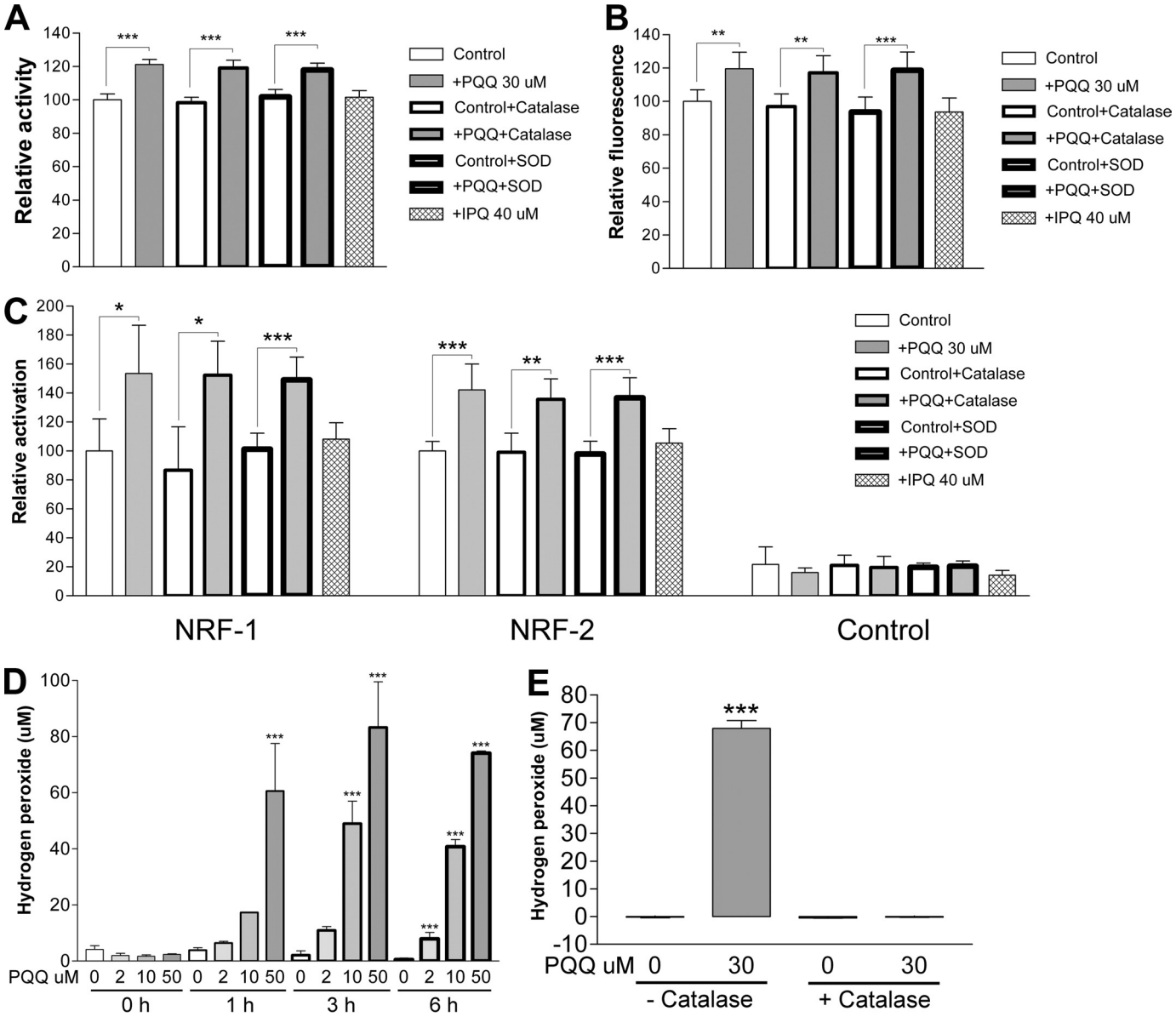

FIGURE 6. **PQQ-induced mitochondrial biogenesis and nuclear respiratory factor activation is not caused by generation of either hydrogen peroxide, superoxide, or IPQ in the media by PQQ.** Increases in mitochondrial content after PQQ exposure were detected by citrate synthase activity (*A*) and a Mitotracker staining microplate assay (*B*), and activation of NRF-1 and NRF-2 was detected by NRF-1 or NRF-2 response element reporter plasmid (*C*) after PQQ exposure. As specified in the figure, cells were incubated in either control media (*C*) or media supplemented with 30 μM PQQ along with either 20 units of catalase/ml of media or 20 units of superoxide dismutase (*SOD*)/ml of media or 40 μM IPQ. The control plasmid (*B*, *Control*) contains the luciferase gene and thymidine kinase promoter but lacks a response element to either NRF-1 or NRF-2. *Columns* and *error bars* indicate the means ± S.D., respectively. *Asterisks* denote significant difference of experimental treatment from respective control condition (indicated with brackets): *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. Hydrogen peroxide (μM concentration) was generated by varying the concentrations of PQQ at 0, 1, 3, and 6 h (*D*) and in the presence or absence of 30 μM PQQ and 20 units of catalase/ml media after 3 h (*E*). *Columns* and *error bars* indicate the means ± S.D., respectively. *Asterisks* denote the significant difference of experimental treatment from control condition; ***, $p < 0.001$. Results are representative data from experiments performed at least twice.

expression found in livers of mice infected with an adenoviral-based CREB inhibitor (38). Although siRNA-mediated knockdown of either CREB or PGC-1α was sufficient to eliminate PQQ-mediated mitochondrial biogenesis and the reduction of CREB by siRNA reduced PGC-1α expression, it is also possible that part of the PQQ-induced mitochondrial biogenesis may be due to CREB activation without the role of PGC-1α. CREB has been reported to bind to the D-loop of mitochondria in rodent brain tissue and primary neurons (39, 40). Although functions of the activation remain unresolved, the downstream consequences are consistent with the PGC-1α pathway. For example, the functional increases in citrate synthase and cytochrome activity, Mitotracker staining, mitochondrial DNA content, and cellular oxygen consumption occurred with increases in NRF-1 and NRF-2 activation and Tfam, TFB1M, and TFB2M mRNA expression. Although other nuclear co-activators related to PGC-1α exist, such as PGC-1β and PGC-1-related co-activator, evidence showing that siRNA-mediated PGC-1α knockdown is sufficient to reduce induction of mitochondrial biogenesis by PQQ suggests that the other co-activators are not involved, although this possibility cannot be excluded. CREB has been shown to regulate PGC-1α, and data shown here sug-

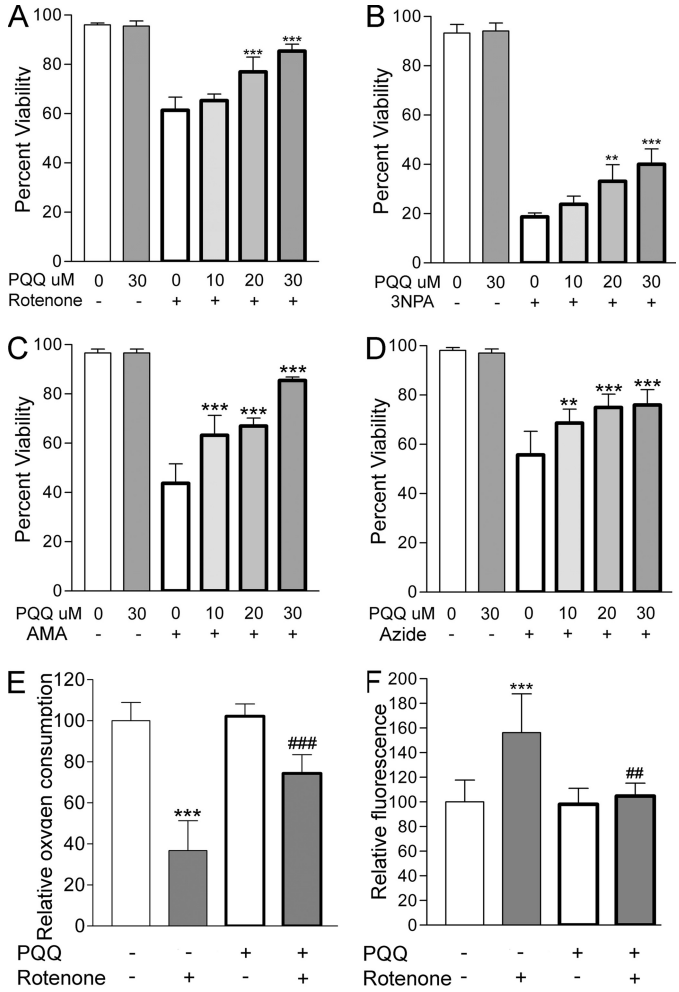

FIGURE 7. **PQQ improves cell viability and preserves mitochondrial function due to mitochondrial inhibitors.** Cell viability was detected by trypan blue dye exclusion after rotenone (*A*), 3-nitropropionic acid (*3NPA*) (*B*), antimycin (*AMA*) (*C*), or sodium azide (Azide) (*D*) exposure. Cells were incubated in control media or media supplemented with 10, 20, or 30 μM PQQ for 24 h followed by exposure to control media or media containing 2 μM rotenone, 40 mM 3-nitropropionic acid, 1 μM antimycin A, or 5 mM sodium azide for 24 h. *Columns* and *error bars* indicate the means ± S.D., respectively. *Asterisks* denote significant difference from control conditions: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. Oxygen consumption (*E*) after PQQ and rotenone exposure was measured by microplate oxygen assay. Cells were incubated in control media or media supplemented with 30 μM PQQ for 24 h, then incubated in control media (with 0.004% DMSO) or media containing 0.2 μM rotenone for 24 h. Oxygen assay was determined by increased fluorescence and is expressed as the difference in relative fluorescence units from 10 to 20 min after the assay start relative to the control conditions (without PQQ or rotenone (contains 0.004% DMSO)). *Columns* and *error bars* indicate the means ± S.D., respectively. *Asterisks* denote significant difference from control conditions (without PQQ or rotenone); ***, $p < 0.001$. *Pound signs* denote significant difference from rotenone without PQQ; ###, $p < 0.001$. Reduction in mitochondrial superoxide by rotenone after PQQ incubation (*F*) was determined by microplate MitoSOX assay. Cells were incubated in control media or media supplemented with 30 μM PQQ for 24 h, then incubated in control media (with 0.25% DMSO) or media containing 20 μM rotenone for 1 h. Mitochondrial superoxide increased fluorescence and was expressed relative to the control conditions (without PQQ or rotenone, contains 0.25% DMSO). *Columns* and *error bars* indicate the means ± S.D., respectively. *Asterisks* denote significant difference from control conditions (without PQQ or rotenone); ***, $p < 0.001$. Pound signs denote significant difference from rotenone without PQQ; ## $p < 0.01$. Results are representative data from experiments performed at least twice.

gest that PQQ acts through CREB to regulate PGC-1α, but regulation of the PGC-1β and PGC-1-related co-activator by CREB has not yet been demonstrated. Also, it is recognized that the PGC-1 family of co-activators likely act and respond to differing physiological stimuli and processes (41).

The observed increase in cell viability after PQQ incubation and mitochondrial inhibition by rotenone is greater than the observed increase in mitochondrial function by PQQ supplementation compared with basal culturing conditions. For example, the increases in mitochondrial parameters after PQQ incubation (30 μM for 24 h) range from 14% for Mitotracker staining to 23% for cellular oxygen respiration. For comparison, the same concentration and duration of PQQ leads to a 39% increase in cell viability and 134% increase in cellular oxygen respiration after rotenone exposure. Adipocyte cell lines established from PGC-1α-deficient mice show modest reductions in mitochondrial genes without significant impairment of brown adipocyte differentiation (42). Adenoviral mediated-expression of PGC-1α in vascular endothelial cells, resulting in a multiple-fold increase in PGC-1α protein expression, leads to a 50% increase in mitochondrial biogenesis (43). Overexpression of PGC-1β in C2C12 muscle cells results in a higher respiration rate compared with overexpression of PGC-1α (44). These studies show that alterations in PGC-1α expression have comparatively modest changes in mitochondrial biogenesis and are consistent with our observations linking PQQ to PGC-1α activity. It is likely that the rather modest reductions in mitochondrial biogenesis may be due to compensation by PGC-1β. The greater ability of PQQ to maintain and protect mitochondrial function against mitochondrial inhibition compared with increased mitochondrial activity relative to basal conditions may be because of a number of reasons. One possibility is that the stimulation of the mitochondrial biogenesis pathway facilitates recovery of mitochondria from damage induced by mitochondrial inhibition. Overexpression of PGC-1α in primary renal cells after oxidant exposure accelerates recovery of mitochondrial function (45). Another possibility is that PQQ protects against reactive oxygen species, which can also be related to PGC-1α activation and protection from the action of mitochondrial inhibitors and reactive oxygen species (16, 43, 46). PQQ has been shown to protect neuroblastoma cells from 6-hydroxydopamine toxicity (47, 48), possibly by preserving DJ-1 activity by preventing the oxidation of cysteines important for DJ-1 function (48). Consequently, it is likely that several mechanisms are responsible for the cytoprotective property of PQQ. Protection from respiratory inhibitors by PQQ occurred by exposing cells to PQQ before the addition of the toxins, and PQQ was removed from the media before the application of the respiratory inhibitors. Unlike when oxygen consumption is measured immediately after PQQ incubation, we did not observe an increase in oxygen consumption after PQQ-supplemented media was replaced with control media for 24 h when determining the effects of PQQ and rotenone on oxygen consumption. This observation suggests that stimulation of mitochondrial biogenesis by PQQ can be transient and reversible. PQQ reverses inhibition along many parts of the mitochondrial oxidation phosphorylation complex, and many mitochondrial disorders show decreased mitochondrial function and increased reactive oxygen species production (49, 50). Previously we showed that PQQ-deficient animals are sensitive to diphenylene iodonium, which is a Complex I inhibitor and anti-

glycemic agent (1). Complex I and III are known to generate superoxide in the mitochondria (51), and inhibition of Complex I, II, III, or IV of the mitochondrial respiratory chain results in an increase in mitochondrial reactive oxygen species and oxidative damage (16, 52–54). An improvement in oxygen utilization and flux (55) may explain how PQQ can prevent an increase in mitochondrial superoxide, which may be a mechanism by which PQQ can protect cells against respiratory inhibition.

The induction of mitochondrial biogenesis by PQQ has a number of health implications. PGC-1α elevation, particularly in muscle and adipose tissue, may also be helpful in that PGC-1α expression is decreased in obesity (56, 57). CREB null and PGC-1α null mice have hepatic steatosis and impaired gluconeogenesis and β-oxidation (38, 58, 59). PQQ-deficient mice have elevated serum triglycerides, which is reversed upon PQQ repletion (1). In addition, mice with deletion of all CREB isoforms have reduced commissural structure formation and impaired fetal T cell development (60), and other mouse models of CREB-targeted deletion show impaired memory and neurodegeneration (61, 62). Likewise, dietary PQQ deprivation results in immune dysfunction (63). PQQ is also neuroprotective when administered by intraperitoneal injection (64, 65) or diet supplementation (66).

Although other phytochemicals are associated with the activation of cell signaling pathways important to mitochondrial function, PQQ has properties that set it apart from other compounds. As an example, resveratrol and genisten have been demonstrated to affect cell-signaling pathways, including those important for mitochondrial biogenesis. Resveratrol can induce deacetylation of PGC-1α (2) and AMP-activated protein kinase activation (2), which are potential mechanisms for PGC-1α activation. Both resveratrol and genistein are relatively insoluble in water, and increasing its water solubility does not increase resveratrol absorption (67), although genistein bioavailability can be increased by complexing genistein with cyclodextrins (68). In contrast, PQQ is relatively water-soluble (>1 g of PQQ/liter of water) and is easily absorbed at low dietary concentrations intakes (69). Although genistein can induce PGC-1α protein expression and mitochondrial biogenesis (3), genistein may also have phytoestrogenic properties because of its ability to activate the estrogen receptor (70).

The observed effects of PQQ are also observed at concentrations lower than those for resveratrol and genistein, particularly *in vivo*. In cell cultures *in vitro*, PQQ causes changes in mitochondriogenesis and function at concentrations similar to those reported recently for small molecule activators of SIRT1 (71), which are being explored for their therapeutic potential (72). These observations suggest that further study related to PQQ is warranted. One important note is that PQQ can increase PGC-1α mRNA transcription, which is different from the post-translation regulation of PGC-1α by resveratrol and raises the likelihood that a combination of various compounds, such as are often present in fruits and vegetables, can stimulate mitochondrial biogenesis through different modes of action. Because mitochondria function as the principal energy source of the cell, compromised function of this key organelle is linked to numerous diseases and metabolic disorders (9, 41). In this regard, PQQ would appear to have therapeutic potential similar to resveratrol, genistein, hydroxytyrosol, quercetin, or other compounds that can induce mitochondrial biogenesis.

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