Resveratrol and para-coumarate serve as ring precursors for coenzyme Q biosynthesis

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Abstract  Coenzyme Q (Q or ubiquinone) is a redox-active polyisoprenylated benzoquinone lipid essential for electron and proton transport in the mitochondrial respiratory chain. The aromatic ring 4-hydroxybenzoic acid (4HB) is commonly depicted as the sole aromatic ring precursor in Q biosynthesis despite the recent finding that para-aminobenzoic acid (pABA) also serves as a ring precursor in Saccharomyces cerevisiae Q biosynthesis. In this study, we employed aromatic [13C6]ring-labeled compounds including [13C6]-4HB, [13C6]-pABA, [13C6]-resveratrol, and [13C6]-coumarate to investigate the role of these small molecules as aromatic ring precursors in Q biosynthesis in Escherichia coli, S. cerevisiae, and human and mouse cells. In contrast to S. cerevisiae, neither E. coli nor the mammalian cells tested were able to form [13C6]-Q when cultured in the presence of [13C6]-pABA. However, E. coli cells treated with [13C6]-pABA generated [13C6]-ring-labeled forms of 3-octaprenyl-4-aminobenzoic acid, 2-octaprenyl-aniline, and 3-octaprenyl-2-aminophenol, suggesting UbiA, UbiD, UbiX, and UbiI are capable of using pABA or pABA-derived intermediates as substrates. E. coli, S. cerevisiae, and human and mouse cells cultured in the presence of [13C6]-resveratrol or [13C6]-coumarate were able to synthesize [13C6]-Q. Future evaluation of the physiological and pharmacological responses to dietary polyphenols should consider their metabolism to Q. —Xie, L.X., K. J. Williams, C. H. He, E. Weng, S. Khong, T. E. Rose, O. Kwon, S. J. Bensinger, B. N. Marbois, and C. F. Clarke. Resveratrol and para-coumarate serve as ring precursors for coenzyme Q biosynthesis. J. Lipid Res. 2015. 56: 909–919.

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Coenzyme Q (Q or ubiquinone) is a polyisoprenylated benzoquinone lipid essential for electron and proton transport in the mitochondrial respiratory chain and in the plasma membrane of Escherichia coli (1, 2). The hydroquinone or reduced form [coenzyme QH2 or ubiquinol (QH2)] functions as a chain-terminating lipid antioxidant and as a coantioxidant to recycle vitamin E (3). Q is also involved in many other metabolic processes, including fatty acid β-oxidation, sulfide oxidization, disulfide bond formation, and pyrimidine metabolism (4–7). Q is composed of a fully substituted benzoquinone ring that is attached to a polyisoprenyl tail with a variable number of isoprenyl units (six for Saccharomyces cerevisiae, eight for E. coli, nine for mouse, and ten for human, hence Qn).

Most cells rely on de novo synthesis for sufficient amounts of Q, although brown adipose tissue was recently discovered to rely on uptake of exogenously supplied Q (8). In baker’s yeast, S. cerevisiae, at least 13 gene products, Coq1-Coq11, Arh1, and Yah1 (9–14) are essential for Q biosynthesis. The Coq1 polypeptide synthesizes the polyisoprenyl tail and Coq2 attaches the tail to the aromatic ring (Fig. 1). The other Coq polypeptides catalyze ring modifications including O-methylation (Coq3), G-methylation (Coq5), hydroxylation (Coq6 and Coq7), and the function of Coq6 requires ferredoxin (Yah1) and ferredoxin reductase (Arh1) (9). The roles of Coq4, Coq8, Coq9, Coq10, and Coq11 have not yet been determined, although they are all required for efficient yeast Q biosynthesis. Schemes of Q biosynthesis generally depict 4-hydroxybenzoic acid (4HB) as the biosynthetic aromatic ring precursor of Q (4). 4HB is considered to derive from chorismate in yeast and from phenylalanine or tyrosine in

Abbreviations  [13C6]-pABA, para-aminobenzoic acid; DoD, drop out dextrose; 4HB, 4-hydroxybenzoic acid; LB, Luria broth; OA, 2-octaprenyl-aniline; OAB, 3-octaprenyl-4-aminobenzoic acid; OAP, 2-amino-3-octaprenylphenol; OP, 2-octaprenyl phenol; pABA, para-aminobenzoic acid; p-coumarate, para-coumarate; Q, coenzyme Q or ubiquinone; QH2, coenzyme QH2 or ubiquinol; Qn, coenzyme Q with n number of isoprene units in the polyisoprenoid tail; RP-HPLC-MS/MS, reverse phase-HPLC-MS/MS; TFA, trifluoroacetic acid.

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animal cells (15–17). Yeast can also use \textit{para}-aminobenzoic acid (pABA) as an alternate ring precursor in the biosynthesis of Q (9, 18). This finding was surprising because pABA is a well-known precursor of folate, which is synthesized de novo by many microorganisms and folate is a vitamin for humans. A biosynthetic scheme was reported recently including proposed steps for the conversion of pABA to Q6 in \textit{S. cerevisiae} (19).

The biosynthesis of Q6 in \textit{E. coli} requires IspB (which synthesizes the octaprenyl diphosphate tail precursor) and 11 Ubi polypeptides (UbiA–UbiJ and UbiX; Fig. 1) (21). UbiC carries out the first committed step in the biosynthesis of Q8, the conversion of chorismate to 4HB (22). UbiA adds the octaprenyl tail to the 4HB ring, followed by the decarboxylation catalyzed by UbiD and UbiX. UbiI adds the first hydroxyl group at the C5 position, followed by O-methylation catalyzed by UbiG, the homolog of yeast Coq3. Additional ring modifications catalyzed by UbiH, UbiE, UbiF, and UbiG generate the final product of Q8. UbiB, an atypical protein kinase similar to animal cells (15–17).
to Coq8, and Ubil play essential, but unknown, functions in E. coli Q8 biosynthesis (21).

Recently, Block et al. (15) identified para-coumarate (p-coumarate) as a ring precursor of Q biosynthesis in Arabidopsis thaliana. Arabidopsis converts phenylalanine to p-coumarate in the cytosol, and following transport into peroxisome, p-coumarate is ligated to CoA and the three-carbon side chain is shortened via peroxisomal β-oxidation (15). Plant peroxisomes appear to contain thiolas and CoA thiolases that can ultimately produce 4HB from 4-hydroxybenzoyl-CoA (15). Tyrosine can also supply the p-coumarate to track their metabolic fate as potential Q biosynthetic precursors in p-coumarate to track their metabolic fate as potential Q biosynthesis (21).

Yeast growth and stable isotope labeling

Solid plate medium included the stated components plus 2% Bacto agar. Yeast colonies from YPD plate medium were first inoculated into 250 ml flasks containing 70 ml YPD medium. Following overnight incubation with shaking (250 rpm) at 30°C, yeast cells were transferred into fresh drop out dextrose (DoD) medium (18). DoD medium contained 2% dextrose, 6.8 g/l Bio101 yeast nitrogen base minus pABA minus folate with ammonium sulfate (MP Biomedicals), and 5.83 mM sodium monophosphate (pH adjusted to 6.0 with NaOH). Amino acids and nucleotides were added as described previously (18).

Stable isotope-containing compounds included β-hydroxy-[aromatic-13C6]benzoic acid ([13C6]4HB) from Cambridge Isotope Laboratories (Andover, MA); resveratrol-[4-hydroxynaphthyl-13C6] ([13C6]resveratrol), and p-amino-[aromatic-13C6]benzoic acid ([13C6]pABA) from Sigma/ISOTEC (Miamisburg, OH). During this work, we discovered that preparations of [13C6]pABA supplied by Cambridge Isotope Laboratories were contaminated with approximately 1% [13C5]4HB. This small level of contamination confounded the initial labeling studies we performed. All studies reported here were performed with the [13C6]pABA obtained from Sigma/ISOTEC, and there was no detectable contamination with [13C6]4HB present in either the [13C6]pABA or [13C6]resveratrol (data not shown).

13C6-labeled aromatic ring precursors were added to fresh DoD medium and incubated with yeast cells (100 A600) at 30°C for 4 h. Cells were collected by centrifugation and pellets were stored at −20°C. The wet weight of each cell pellet was determined by subtracting the weight of the tube from the total weight. Protein assays (BCA assay, Thermo) were performed on yeast cell lysates (25). For 13C6-coumarate labeling, BY4741 yeast cells were incubated in 5 ml of SD-complete medium at a starting cell density of 0.1 A600 and incubated at 30°C for 24 h. The yeast cell density after incubation was approximately 6 A600.

Synthesis of p-coumaric acid [aromatic-13C6]

The synthesis was similar to the method described by Robbins and Schmidt (26), with the following modifications. To a flame-dried flask (25 ml) was added 4-hydroxybenzaldehyde [aromatic-13C6] (50 mg), malonic acid (75 mg), piperidine (5 µl), and pridine (1 ml). The reaction mixture was stirred under argon at 92°C. The reaction was monitored through thin layer chromatography on 0.25 mm SiliCycle silica gel plates and visualized under UV light and with permanganate or 2,4-dinitrophenylhydrazine staining. Upon completion (12 h), the mixture was sequentially added to 10 ml water, neutralized to pH 7–8, and then washed with dichloromethane. The aqueous solution was acidified to pH 1 and then extracted twice using ethyl acetate. The combined organic extract was concentrated in vacuo and purified through flash column chromatography. Flash column chromatography was performed with SiliCycle Silica-P Flash silica gel (60 Å pore size, 40–63 µm) and 50% ethyl acetate in hexanes as mobile phase, to furnish an off-white solid (38 mg, 87% yield). A portion was further purified by semi-preparative RP-HPLC (Waters SunFire C18,

### MATERIALS AND METHODS

#### Yeast growth and stable isotope labeling

The S. cerevisiae strains used are described in Table 1. YPD medium (2% glucose, 1% yeast extract, 2% peptone) was prepared as described (24). Solid plate medium included the stated components plus 2% Bacto agar. Yeast colonies from YPD plate medium were first inoculated into 250 ml flasks containing 70 ml YPD liquid medium. Following overnight incubation with shaking (250 rpm) at 30°C, yeast cells were transferred into fresh drop out dextrose (DoD) medium (18). DoD medium contained 2% dextrose, 6.8 g/l Bio101 yeast nitrogen base minus pABA minus folate with ammonium sulfate (MP Biomedicals), and 5.83 mM sodium monophosphate (pH adjusted to 6.0 with NaOH). Amino acids and nucleotides were added as described previously (18).

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10 × 250 mm, 5 μm) using the following gradient elution (solvent A: water + 0.1% trifluoroacetic acid (TFA); solvent B: acetonitrile + 0.1% TFA, flow rate 6.0 ml/min): 0–2 min 10% B; 2–20 min linear 10–45% B; 20–22 min linear 45–10% B; 22–25 min 10% B. Fractions were pooled, concentrated in vacuo, and the aqueous remainder was lyophilized to give a white powder (11.8 mg). RP-HPLC analysis indicated >99% purity at 210 and 254 nm, and no detectable 4HB (Waters Sunfire C18, 4.6 × 250 mm, 5 μm; solvents A/B as above, flow rate 1.00 ml/min) using the following gradient elution: 0–1 min 10% B; 1–20 min linear 10–100% B; 20–25 min 100% B; 25–27 min linear 100–10% B; 27–30 min 10% B.

NMR spectra were recorded using a Bruker Avance-500 spectrometer, calibrated to residual acetone-d₆ as the internal reference (2.05 ppm for ¹H NMR; 29.9 and 206.7 ppm for ¹³C NMR). ¹H NMR spectral data are reported in terms of chemical shift (δ, parts per million), multiplicity, coupling constant (hertz), and integration. ¹³C NMR spectral data are reported in terms of chemical shift (δ, parts per million), multiplicity, and coupling constant (hertz). The following abbreviations indicate the multiplicities: s, singlet; d, doublet; t, triplet; m, multiplet; br, broad. ¹H NMR (500 MHz, acetone-d₆) δ 9.40 (br s, 1H), 7.70–7.65 (m, 1H), 7.65–7.57 (m, 1H), 7.39–7.35 (m, 1H), 7.05–7.02 (m, 1H), 6.71–6.69 (m, 1H), 6.32 (dd, J = 5.2, 16.7 Hz, 1H) (supplementary Fig. 1A); ¹³C NMR (125 MHz, acetone-d₆) δ 159.5 (dt, J = 64.8, 8.6 Hz), 129.9 (tt, J = 58.8, 4.4 Hz), 125.9 (dt, J = 58.0, 9.2 Hz), 115.6 (dt, J = 64.6, 4.1 Hz) (supplementary Fig. 1B). GC-MS data were recorded using an Agilent 6890-5975 GC mass spectrometer equipped with an autosampler and an HP5 column; the sample was dissolved in methanol followed by 1.8 ml of petroleum ether. Q₈ was added as an internal standard for the determination of Q₈ content in.

E. coli growth and stable isotope labeling

E. coli strains are described in Table 1. The BW25113 ΔubiC::kan mutant strain was obtained from the Keio collection (27). Phage P1 was used to transduce the mutation into the MG1655 strain, yielding MG1655ubiC. The replacement of the chromosomal ubiC gene by the kan gene was checked by PCR amplification. Cells were inoculated in 100 ml of Luria broth (LB) for 16 h at 37°C. Cells (50 A₆₀₀) from each sample were collected by centrifugation, and the collected pellets were resuspended in fresh LB medium in the presence of either 10 μg/ml of ¹³C₆-pABA, ¹³C₆-resveratrol, and incubated at 37°C with shaking at 250 rpm. Incubations with vehicle control contained an equivalent volume of ethanol (in all conditions the final ethanol concentration was 0.2%). Cells were collected by centrifugation after 4 h and stored at −20°C for LC-MS/MS lipid analyses. For ¹³C₆-coumarate labeling, HW272, HW25113, MG1655, and MG1655ubiC were inoculated in 5 ml of LB for 16 h at 37°C (MG1655ubiC was incubated in LB with 50 μg/ml kanamycin). Cells were diluted to 0.2 A₆₀₀ in fresh media with 15 μg/ml of ¹³C₆-coumarate and incubated for 24 h. Cells were pelleted for lipid extraction and LC-MS/MS analyses.

Animal cell culture and stable isotope labeling

U251 human glioma and 3T3 mouse fibroblast cells were cultured in DMEM (Gibco). U87 human glioma cells were cultured in Iscove’s Modified Dulbecco’s Medium (Gibco). Human embryonic kidney 293T cells were cultured in DMEM with 1 mM sodium pyruvate (Gibco). All cells were passaged in the stated media supplemented with 10% FBS (Omega Scientific) and 1% penicillin-streptomycin (10,000 U/ml) (Life Technologies). Equal numbers of cells were plated approximately 12 h prior to treatment experiments. During treatment with stable isotope-labeled compounds, cells were cultured with 1% FBS, unless otherwise stated. Cells were cultured with the designated stable isotope-labeled compound for 24 h, then washed with PBS [137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 2 mM KH₂PO₄ (pH 7.4)], and released from the culture dish with 0.25% trypsin-EDTA (Gibco). Aliquots of the released cells were stained with Trypan blue and the number of cells counted with the Cellometer Auto T4 (Nexcelom Bioscience); aliquots (5%) were also removed for determination of protein content (BCA assay; Thermo). The remaining cells in the suspension were collected by centrifugation. Cell pellets were stored at −20°C.

Lipid extraction

Cell pellets were thawed on ice and then suspended in 1.2 ml of methanol followed by 1.8 ml of petroleum ether. Q₈ was added as an internal standard for the determination of Q₈ content in.
yeast lipid extracts. Diethoxy-Q_{10} (28) was used as an internal standard for determination of Q_{6} and Q_{10} in mammalian cell lipid extracts and Q_{6} in E. coli cell lipid extracts. Samples were vortexed for 45 s, then the upper layer was removed to a new tube, and another 1.8 ml of petroleum ether was added to the lower phase and the sample was vortexed again for 45 s. The upper layer was again removed and combined with the previous organic phase. The combined organic phase was dried under a stream of nitrogen gas and resuspended in 200 μl of ethanol (USP; Aaper Alcohol and Chemical Co., Shelbyville, KY).

**RP-HPLC-MS/MS**

The RP-HPLC-MS/MS analyses were performed as previously described for determination of Q_{6} in yeast lipid extracts (11, 18) and determination of Q_{6} and Q_{10} in mammalian lipid extracts (28, 29). Briefly, a 4000 QTRAP linear MS/MS spectrometer from Applied Biosystems (Foster City, CA) was used. Applied Biosystem software, Analyst version 1.4.2, was used for data acquisition and processing. A binary HPLC solvent delivery system was used with either a Luna phenyl-hexyl column (particle size 3 μm, 100 × 4.60 mm; Phenomenex) for yeast cell lipid extracts or a Luna phenyl-hexyl column (particle size 3 μm, 50 × 2.00 mm; Phenomenex) for mammalian and bacteria cell lipid extracts. The mobile phase consisted of solvent A (methanol:isopropanol, 95:5, with 2.5 mM ammonium formate) and solvent B (isopropanol, 2.5 mM ammonium formate). For separation of yeast quinones, the percentage of solvent B increased linearly from 0 to 5% over 6 min, and the flow rate increased from 600 to 800 μl/min. The flow rate and mobile phase were linearly changed back to initial condition by 7 min. For separation of bacteria and mammalian quinones, the percentage of solvent B for the first 1.5 min was 0%, and increased linearly to 10% by 2 min. The percentage of solvent B remained unchanged for the next min and decreased linearly back to 0% by 6 min. A constant flow rate of 800 μl/min was used. All samples were analyzed in multiple reaction monitoring mode; multiple reaction monitoring transitions were as follows: m/z 591/197.1 (Q_{6}); m/z 610/197.1 (Q_{6}H_{2} with ammonium adduct); m/z 597/203.1 (^{13}C_{6}Q_{6}); m/z 616/203.1 (^{13}C_{6}Q_{6}H_{2} with ammonium adduct); m/z 636/106 [2-octaprenyl-aniline (OA)]; m/z 637/107 [2-octaprenyl phenol (OP)]; m/z 642/112 (^{13}C_{6}-OA); m/z 643/113 (^{13}C_{6}-OP); m/z 652/122 [2-amino-3-octaprenylphenol (OAP)]; m/z 658/128 (^{13}C_{6}-OAP); m/z 682/150

![Fig. 3. E. coli cells produce ^{13}C_{6}-pABA-derived octaprenyl-products. HW272, MG1655, and MG1655ubiC cells were cultured and processed as described in the Materials and Methods. Bar plots show the total content of OAB (A), OA (B), OP (C), and OAP (D). Each bar represents mean ± SD. The black bar of each column represents the designated ^{13}C_{6}-labeled intermediate, while the white bar represents the ^{12}C-intermediate. Each y axis represents the area under the peak of interest first normalized by the internal standard (diethoxy-Q_{10}), and then by the value of OD_{600} of the extracted cell pellets.](image-url)
pABA is a demonstrated ring precursor of Q biosynthesis in the yeast S. cerevisiae (9, 18), but is not utilized as a ring precursor of Q biosynthesis in Arabidopsis (15). To investigate whether pABA may serve as a ring precursor of Q biosynthesis in mammalian cells, human U251 cells were cultured in the presence of 7, 70, or 700 μM of either 13C6-4HB or 13C6-pABA for 24 h prior to RP-HPLC/MS/MS analysis of Q content (Fig. 2A). U251 cells readily converted 13C6-4HB to 13C8-Q10, however, incubations with 13C6-pABA produced no detectable 13C6-Q10 (Fig. 2A, supplementary Fig. 2). Treatments of U251 cells with various 13C6-4HB concentrations did not alter the total Q10 content; however incubation with 700 μM 13C6-pABA resulted in significantly lower total Q10 content in mammalian cells (P < 0.05).

To examine whether pABA is utilized as a ring precursor in E. coli Q8 biosynthesis, 13C6-pABA or 13C6-4HB was added to cultures of the designated E. coli strains. HW272 and MG1655 are wild-type strains, while MG1655ubiC contains a deletion of the ubiC gene encoding chorismate prenyltransferase pyruvate lyase (Table 1). Each E. coli strain was cultured in LB medium with aromatic ring precursors added to a final concentration of 10 μg/ml (Fig. 2B). Each of the E. coli strains incubated in the presence of 10 μg/ml 13C6-4HB accumulated significant amounts of 13C8-Q6. No incorporation of 13C6-pABA into 13C6-Q8 was detected with the wild-type strains. Interestingly, the E. coli ubiC mutant was also unable to use pABA to synthesize Q6. This result suggests that pABA is not utilized, even under conditions of impaired 4HB synthesis.

Detection of various polyprenylated derivatives of 13C6-pABA indicated that the E. coli strains tested were able to take up this ring precursor. For example, 13C6-octaprenyl-K-anilinobenzoic acid (OAB) indicated that 13C6-pABA-treated E. coli cells successfully absorbed 13C6-pABA from the medium and performed the ring prenyltransferase step catalyzed by UbiA (30) (Fig. 3A). 13C6-OA (Fig. 3B) was also readily detected in lipid extracts of the 13C6-pABA-treated E. coli cells. Notably, the ubiC mutant accumulated significantly more aniline-containing intermediates (Fig. 3A, B), even in the absence of pABA addition, presumably due to a deficiency in 4HB synthesis. The product 13C6-OAP (Fig. 3D) was also observed in 13C6-pABA-treated E. coli cells and is probably due to UbiD, which catalyzes the first hydroxylation step in Q8 biosynthesis (31). The ubiC mutant accumulated 10 times more 13C-OAP than HW272 or MG1655, a finding independent of the supplied 13C6-pABA, suggesting that OAP might be a “dead-end” product. 13C6-OAB, 13C6-OA, or 13C6-OAP were not detected in either the 13C6-4HB-treated or control E. coli cells (Fig. 3A, B, D). 13C6-QP was detected only in 13C6-4HB-treated cells (Fig. 3C). These results suggest that although pABA is prenylated and can be further modified by UbiD, UbiX, and UbiE, E. coli may not be able to process the aniline-containing ring intermediates to later intermediates or to Q6.

Given that S. cerevisiae can utilize either 4HB or pABA in Q6 biosynthesis, we investigated the use of other possible aromatic ring precursors. Surprisingly, wild-type yeast could use resveratrol as a ring precursor in the synthesis of Q6 (Fig. 4). W303 cells cultured in the presence of 68, 282, or 974 μM of 13C6-resveratrol showed increasing amounts of 13C6-Q6, while the ethanol control samples contained no detectable 13C6-Q6. Notably, the increased amount of resveratrol did not alter the total Q6 content. We next examined whether human or mouse cells could use resveratrol as a ring precursor to Q6. The three human cell lines we examined were able to convert resveratrol to Q6 as shown by the accumulation of 13C6-Q10 (Fig. 5A–C, supplementary Fig. 3A–C). 13C6-Q6 (Fig. 5D, supplementary Fig. 3D) also accumulated in mouse 3T3 fibroblasts, when cultured in the presence of 70 μM of 13C6-resveratrol. Although cells cultured with 13C6-4HB accumulated significantly more 13C6-Q10 than when cultured with 13C6-resveratrol, the incorporation of 13C6-resveratrol into 13C6-Q10 accounted for approximately 10% of the total Q10, a proportion that was much higher than that observed in wild-type yeast cells (the 13C6-Q6 was less than 1% of the total Q6). 13C6-Q6 content increased in response to the

**RESULTS**

13C6-resveratrol is a ring precursor to Q6 biosynthesis in S. cerevisiae. Yeast W303B wild-type cells incubated with 0, 68, 282, or 974 μM of 13C6-resveratrol were cultured and analyzed as described in the Materials and Methods. The gray bar represents 13C6-Q6, and the white bar indicates 13C6-Q8, which was used as the internal standard.
increasing concentrations of $^{13}$C$_6$-resveratrol, while the total Q content again remained unaltered (Fig. 5E, F). Unfortunately, higher concentration (>70 μM) of resveratrol induced cell death, thus we were not able to examine the amount of $^{15}$C$_6$-Q synthesized in the presence of higher $^{13}$C$_6$-resveratrol concentrations.

E. coli also utilized resveratrol as an alternative ring precursor to Q, although to a lesser extent when compared with yeast, mouse, or human cells. HW272 and MG1655 cells cultured in LB medium in the presence of 10 μg/ml $^{13}$C$_6$-resveratrol accumulated trace amounts of $^{13}$C$_6$-Q$_8$ (Fig. 6A, supplementary Fig. 4A). In comparison, 10 μg/ml of $^{13}$C$_6$-4HB resulted in $^{13}$C$_6$-labeling of more than two-thirds of the total Q content in the same cells. However, the E. coli ubiC mutant, with a defect in de novo synthesis of 4HB, produced significantly more $^{13}$C$_6$-Q$_8$ when treated with $^{13}$C$_6$-resveratrol (Fig. 6A, supplementary Fig. 4A).

Fig. 5. Human and mouse cells utilize $^{13}$C$_6$-resveratrol as a ring precursor in Q biosynthesis. U251 cells (A); U87 cells (B); 293T cells (C); and 3T3 cells (D) were cultured in medium with 1.0% FBS in the presence of either $^{13}$C$_6$-4HB (278 μM), $^{13}$C$_6$-resveratrol (70 μM), or ethanol as vehicle control for 24 h prior to collection. Increasing concentrations of resveratrol does not alter total Q levels in mammalian cells: U251 (E) and 3T3 (F) cells cultured in the presence of 0, 7, 24, or 68 μM $^{13}$C$_6$-resveratrol were processed and analyzed. Collected cells were extracted and analyzed by RP-HPLC-MS/MS as described in the Materials and Methods. The gray bars represent $^{13}$C$_6$-Q, and the white bars represent $^{12}$C-Q. Error bars represent SD (n = 4). Diethoxy Q$_{10}$ was used as an internal standard.

Given the structural similarity of resveratrol with p-coumarate, we tested the ability of yeast to utilize $^{13}$C$_6$-coumarate as a ring precursor of $^{13}$C$_6$-Q$_6$. Yeast wild-type BY4741 cells were cultured in SD-complete medium with 7, 70, or 700 μg/ml $^{13}$C$_6$-coumarate for 24 h (Fig. 7A). We found that while the total amount of Q$_6$ did not change with different amounts of $^{13}$C$_6$-coumarate, the amount of $^{13}$C$_6$-Q$_6$ increased with higher concentrations of $^{13}$C$_6$-coumarate, although the incorporation was lower as compared with $^{13}$C$_6$-4HB. U251 human cells were labeled with increasing concentrations of $^{13}$C$_6$-resveratrol, while the total Q content again remained unchanged (Fig. 5E, F).

Fig. 4B. $^{13}$C$_6$-OP was detected only when E. coli strains were cultured in the presence of $^{13}$C$_6$-4HB, and not with $^{13}$C$_6$-resveratrol (Fig. 6B), suggesting that step(s) at which resveratrol is used as a ring precursor may not depend on its conversion to 4HB, or that the production of 4HB from resveratrol is slow compared with the step where OP is utilized.

E. coli also utilized resveratrol as an alternative ring precursor to Q, although to a lesser extent when compared with yeast, mouse, or human cells. HW272 and MG1655 cells cultured in LB medium in the presence of 10 μg/ml $^{13}$C$_6$-resveratrol accumulated trace amounts of $^{13}$C$_6$-Q$_8$ (Fig. 6A, supplementary Fig. 4A). In comparison, 10 μg/ml of $^{13}$C$_6$-4HB resulted in $^{13}$C$_6$-labeling of more than two-thirds of the total Q content in the same cells. However, the E. coli ubiC mutant, with a defect in de novo synthesis of 4HB, produced significantly more $^{13}$C$_6$-Q$_8$ when treated with $^{13}$C$_6$-resveratrol (Fig. 6A, supplementary Fig. 4A).
coumarate is a ring precursor for Q biosynthesis in S. cerevisiae cells could utilize pABA as a ring precursor in Q biosynthesis was rather surprising because pABA is a crucial intermediate in folate biosynthesis (9, 18). The addition of pABA to either E. coli or human cells leads to a concentration-dependent inhibition of Q content (4, 32, 33). Another aromatic ring compound, 4-nitrobenzoic acid, inhibited Q biosynthesis in mammalian cells by competing with 4HB for Coq2 (34). While pABA does not function as a ring precursor of Q in Arabidopsis (15), it remained possible that pABA might still be utilized as a ring precursor in Q biosynthesis in human and E. coli cells. Therefore, we employed $^{13}$C$_{6}$-pABA to investigate its fate in human and E. coli cells.

Treatment of cells with $^{13}$C$_{6}$-pABA revealed that pABA was not an aromatic ring precursor to Q biosynthesis in either human or E. coli cells. In order to rule out the scenario that E. coli cells might utilize pABA as a ring precursor in Q biosynthesis only when the primary ring precursor 4HB is not available, we incubated ubiC mutants, which have defects in the de novo synthesis of 4HB in the presence of $^{13}$C$_{6}$-pABA. However, even ubiC mutants were not able to utilize pABA for Q$_{8}$ biosynthesis. Interestingly, we detected multiple nitrogen-containing intermediates that derived from $^{13}$C$_{6}$-pABA. Detection of $^{13}$C$_{6}$-OAB in all three strains (HW272, MG1655, and ubiC) confirmed $^{13}$C$_{6}$-pABA uptake (Fig. 3A). Further modifications of the $^{13}$C$_{6}$-OAB resulted in $^{13}$C$_{6}$-OA and $^{13}$C$_{6}$-OAP, indicating UbiA, UbiD/UbiX, and Ubi tolerated the amino ring substituent (Fig. 3B, D) (21). OAP accumulated in the ubiC mutant independent of $^{13}$C$_{6}$-pABA addition, suggesting that OAP could be a dead-end product derived from endogenously produced unlabeled pABA. Neither HW272 nor MG1655 wild-type E. coli accumulated significant amounts of OAP, indicating that E. coli cells tend to process pABA through early steps in the Q biosynthetic pathway when 4HB content is low. These observations are consistent with studies that showed an E. coli mutant that lacked chorismate synthase converted pABA to OAB when cultured without addition of 4HB (33). We did not detect further downstream nitrogen-containing Q biosynthetic intermediates using targeted and limited-untargeted LC-MS/MS approaches. However, the presence of additional N-containing Q intermediates downstream of OAP cannot be ruled out.

It was shown that Lithospermum erythrorhizon cell cultures are able to synthesize 4HB from p-coumarate (35) and A. thaliana uses p-coumarate to synthesize Q (15). Therefore, we investigated whether p-coumarate is a ring precursor for Q in different organisms. We found that yeast, E. coli, and human cells can derive Q from p-coumarate. This finding will help us understand how 4HB is generated in these organisms. In A. thaliana, p-coumarate is activated by CoA ligase and the aliphatic chain is shortened to 4HB in peroxisomes (15). Because yeast, human cell cultures, and E. coli can use p-coumarate to make Q, it is possible that these organisms derive 4HB from p-coumarate in a similar manner.

A wide spectrum of activities is attributed to stilbenoids produced by a variety of plants when under attack by pathogens (36). A stilbenoid of recent fame, resveratrol,
Alternate ring precursors in coenzyme Q biosynthesis have led to vigorous research investigating its mechanisms of action. Here we show that resveratrol serves as an aromatic ring precursor in Q biosynthesis in *E. coli*, yeast, and mammalian cells. Wild-type *E. coli* barely utilized resveratrol for Q biosynthesis; however, significant incorporation of the resveratrol ring into Q was observed in *ubiC* mutants. Preferential incorporation of alternate ring precursors in the *E. coli ubiC* mutant strain is presumably due to the defect in synthesis of 4HB. In contrast, approximately 10% of the total Q content in human and mouse acts as a chain-breaking antioxidant, modulates cellular antioxidant enzymes and apoptosis, and has beneficial effects on neurodegenerative and cardiovascular diseases, eliciting metabolic responses similar to dietary restriction (37, 38). Although there is much controversy regarding the lifespan extension effects of resveratrol (39), its effects on age-associated diseases in animal models has generated considerable enthusiasm for research on its mechanism of action (40). Many questions remain regarding resveratrol biodistribution, its metabolism, and the biological effects of resveratrol metabolites (41). The beneficial health effects of resveratrol have led to vigorous research investigating its mechanisms of action.

Here we show that resveratrol serves as an aromatic ring precursor in Q biosynthesis in *E. coli*, yeast, and mammalian cells. Wild-type *E. coli* barely utilized resveratrol for Q biosynthesis; however, significant incorporation of the resveratrol ring into Q was observed in *ubiC* mutants. Preferential incorporation of alternate ring precursors in the *E. coli ubiC* mutant strain is presumably due to the defect in synthesis of 4HB. In contrast, approximately 10% of the total Q content in human and mouse...
cells harbored the ring derived from 13C6-resveratrol after 24 h of incubation. The maximum concentration of resveratrol tested was lower than either 4HB or pABA because resveratrol induced apoptotic cell death (42). Thus, we monitored cell viability in our experiments and limited the amount of resveratrol tested in order to avoid induction of cell death.

The metabolism of resveratrol responsible for its incorporation into Q has not been determined. Animals harbor two carotenoid cleaving enzymes, BCO1 and BCO2, and both are homologs of the carotenoid cleavage oxidase family (43). BCO1 is cytosolic and is responsible for cleaving β-carotene to form two molecules of retinal, while BCO2 is located in the inner mitochondrial membrane and acts on xanthophylls (44). It is tempting to speculate that BCO2, which has broader substrate specificity, might possibly cleave stilbenoids to produce two ring aldehyde products. Other family members of carotenoid cleavage enzyme in bacteria and fungi cleave resveratrol to produce 4-hydroxy-benzaldehyde and 3,5-dihydroxy-benzaldehyde (45, 46). Notably the 4′ hydroxyl group of resveratrol has been identified as crucial for antioxidant and neuroprotective effects of stilbenoids (47). It seems likely that BCO2, which has broader substrate specificity, might possibly cleave stilbenoids to produce two ring aldehyde products. Other family members of carotenoid cleavage enzyme in bacteria and fungi cleave resveratrol to produce 4-hydroxy-benzaldehyde and 3,5-dihydroxy-benzaldehyde (45, 46).

Of the more than one hundred clinical trials testing the efficacy of resveratrol or other polyphenols (clinical trials.gov), few determine the metabolic fate of the administered supplement. When metabolism of resveratrol is studied, the focus is on aqueous soluble polar metabolites of resveratrol, including sulfated and glucuronidated conjugation products (50). The new finding that a metabolic conversion of resveratrol into Q occurs in eukaryotes shows that exogenous antioxidants may be utilized as precursors to synthesize a wholly different class of molecule. The effects of resveratrol in mimicking calorie restriction (37, 38) may be due in part to its conversion to Q, a lipid known to induce anti-inflammatory responses (51), an essential component of mitochondrial energy metabolism, and a potent lipid soluble antioxidant (4). Investigation of the pharmacological responses to diverse dietary polyphenols (e.g., curcumin) should be expanded to include this molecular fate. Further investigation on this subject will give us a better understanding on the origin of the benzenoid moieties of Q in different organisms.

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