Comparison of dietary polyphenols for protection against molecular mechanisms underlying nonalcoholic fatty liver disease in a cell model of steatosis

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Scope: Dietary polyphenols have shown promise in protecting the liver against nonalcoholic fatty liver disease. The relative effectiveness and mechanisms of different polyphenols however is mostly unknown.

Methods and results: In a model of steatosis using HepG2 hepatocytes, we evaluated the protective effects of different classes of polyphenols and the contributing mechanisms. The treatment of the cells with oleic acid increased reactive oxygen species (ROS) generation and expression of tumor necrosis factor alpha (TNF-α), decreased expression of uncoupling protein 2, and decreased mitochondrial content and markers of biogenesis. The treatment with 1–10 μM polyphenols (resveratrol, quercetin, catechin, cyanidin, kuromanin, and berberine), as well as phenolic degradation products (caffeic acid, protocatechuic acid, and 2,4,6-trihydroxybenzaldehyde), all protected by more than 50% against the oleic acid induced increase in ROS. In other mechanisms involved, the polyphenols except anthocyanins strongly prevented or reversed the effect on mitochondrial content/biogenesis, increased expression of manganese superoxide dismutase, and prevented the large increase in TNF-α expression. Most polyphenols also prevented the decrease in uncoupling protein 2. The anthocyanins were unique in decreasing ROS generation without inducing mitochondrial biogenesis or manganese superoxide dismutase expression.

Conclusion: While different polyphenols similarly decreased cellular ROS in this model of steatosis, they differed in their ability to suppress TNF-α expression and induce mitochondrial biogenesis and content.

Keywords: Mitochondrial biogenesis / Nonalcoholic fatty liver disease / Polyphenols / Respiratory proteins / ROS

1 Introduction

Nonalcoholic fatty liver disease (NAFLD) is a prevalent liver disease, affecting 10–35% of the general population in different countries [1] and 75% of obese patients with type 2 diabetes [2, 3]. NAFLD may result in serious complications such as nonalcoholic steatohepatitis (NASH), cirrhosis, and hepatocellular carcinoma. Among possible treatments, dietary polyphenols have been identified to offer a potential therapy for NAFLD and its progression to NASH [4, 5].

The leading hypothesis regarding development and progression of fatty liver disease is a “two-hit” hypothesis [6]. This hypothesis suggests that hepatic accumulation of fats containing oleic acid (OA) [7] is the first hit, which predisposes the liver to second hits such as oxidative stress.
and inflammation. Recently other second hits have been suggested to be involved, including insulin resistance and mitochondrial dysfunction [8, 9], which interplay in a concerted manner to exacerbate simple steatosis to steatohepatitis, cirrhosis, and fibrosis.

Oxidative stress and inflammation are two second hits that may be targeted by polyphenols. Oxidative stress has been demonstrated by numerous studies to have a central role in the liver damage and the progression of NAFLD to NASH and has been associated with lower antioxidant activities [10]. Accordingly, maintaining redox homeostasis by polyphenol supplementation can be a possible strategy to prevent reactive oxygen species (ROS) production in NAFLD [11]. Inflammatory processes also have a major role in the liver damage and activation of collagen-producing hepatic stellate cells leading to fibrosis [12], and polyphenols often show anti-inflammatory effects [5].

Mitochondrial dysfunction is another aspect that could be targeted in therapies involving polyphenols. An accumulating body of evidence suggests NAFLD as a mitochondrial disease due to the abnormality in function and morphology of hepatic mitochondria [10, 13–15]. Correspondingly, mitochondrial dysfunction is correlated with severity of the disease and its progression to NASH [16]. This involvement is due to the pivotal role of mitochondria in lipid metabolism, lipid peroxidation, ROS generation, cytokine production, and apoptosis [17]. Polyphenols such as resveratrol have been observed to stimulate mitochondrial biogenesis in different tissues in a variety of dietary studies [18, 19].

There are many studies on NAFLD in animal models or in vitro showing beneficial effects of polyphenols, including effects on oxidative stress [20, 21], inflammation [21, 22], steatosis [23–26], and mitochondrial biogenesis [19, 27]. However, most are with a single polyphenol or food source, so a major question might be which polyphenols are more effective and by what mechanisms. Therefore, in an in vitro model of steatosis we compared the effects of different classes of polyphenols on ROS generation, tumor necrosis factor alpha (TNF-α) production, and mitochondrial biogenesis to explore the molecular mechanisms underlying NAFLD.

2 Materials and methods

2.1 Materials

Eagle’s minimum essential medium (MEM) for cell culture was obtained from Hyclone (Logan, UT, USA). Fetal bovine serum, antibiotic mixture (penicillin/streptomycin), 2′,7′-dichloro-dihydrofluorescein diacetate (DCFH-DA), fatty acid free BSA, catechin, quercetin, resveratrol, berberine, and OA were purchased from Sigma-Aldrich (USA). Cyanidin, cyanidin-3-glucoside (kuromarin), malvidin, and delphinidin were from Extrasynthese (France). RNAeasy mini kit for isolating RNA was purchased from Qiagen. VILO cDNA synthesis kit, TRIzol reagent, and Power SYBR Green PCR master mix for PCR were purchased from Invitrogen (USA). MitoTracker Green was purchased from Life Technologies (USA).

2.2 HepG2 cell culture

HepG2 cells (human hepatoma cell line) were grown in T75 cell culture flask containing Eagle’s MEM containing 5 mM glucose, 10% fetal bovine serum, and 1% penicillin/streptomycin (100 units/mL penicillin and 100 mg/mL streptomycin) in an incubator containing 5% CO2 at 37°C. When HepG2 cells became 70–80% confluent, cells were subcultured and used for different experiments.

2.3 Treatment with polyphenols and OA/BSA

OA stock (100 mM) was prepared by dissolving in DMSO and the stock was kept at -20°C until use. HepG2 hepatocytes were cultured in MEM at 5 × 10⁴ cells in each well in 96-well optical-bottom plates for 24 h until they reached 80–90% confluence. After 24 h, medium was replaced with MEM without phenol red containing 10% fetal bovine serum as well as 1% fatty acid free bovine serum albumin (BSA), and polyphenols in different final concentrations (1, 5, and 10 µM) were added to related wells. BSA (1%) was used to dissolve OA. After polyphenol pretreatment for 2 h, 1.5 mM OA was added to each well except the untreated control condition.

2.4 Intracellular ROS generation

Intracellular ROS was measured using DCFH-DA. This probe is a cell-permeant nonfluorescent dye. Upon being trapped inside the cells through cleavage of the acetate groups by intracellular deacetylases, ROS oxidize DCFH to 2′,7′-dichlorofluorescein, which is a highly fluorescent compound. After 24 h incubation with (or without) OA, medium was replaced with fresh medium containing DCFH-DA (25 µM final concentration). Hydrogen peroxide (100 µM) was added to related wells as a positive control. After incubating with DCFH-DA at 37°C for 1 h, cells were carefully washed twice with Hank’s balanced salt buffer and fluorescence of each well was read at nine different spots using a plate reader (Biotek) (excitation wavelength 485 nm and emission 528 nm). Each experiment was repeated in triplicate (biological replicates) with three replicate wells (technical replicate) per experiment.

2.5 Mitochondrial content

To determine mitochondrial content, HepG2 hepatocytes were labeled with the mitochondria-specific dye, MitoTracker Green. MitoTracker Green is relatively insensitive to changes
in mitochondrial membrane potential [28], and so its fluorescence is proportional to the cell’s mitochondrial mass [29].

HepG2 cells were seeded (7.5 × 10^4) in each well in 96-well optical-bottom plates for 24 h in MEM medium without BSA. Next day, medium was replaced with MEM containing 1% BSA and cells were pretreated with polyphenols at 10 μM for 2 h followed by OA at 1.5 mM for 24 h. Then, the medium was replaced with fresh medium without FBS containing MitoTracker green at a final concentration of 100 μM followed by incubation at 37°C in the dark for 1 h. Incubation medium was then removed, cells were carefully washed twice with Hanks’ buffer and the average fluorescence intensity was quantified using a plate reader (Biotek) by reading nine different spots in each well (excitation wavelength 485 nm and emission 528 nm). Each experiment was repeated in triplicate with three replicate wells per experiment. Fluorescence images were captured using a ZOE Fluorescent Cell Imager (BioRad, USA).

2.6 RT-PCR

2.6.1 RNA isolation from HepG2 cells

For determining relative mRNA expression, total RNA was isolated from HepG2 cells using TRIzol reagent and RNAeasy mini kit according to the manufacturer’s instructions. For isolating RNA, 1 × 10^6 cells per group were plated in six-well plate for the first 48 h to grow. On the third day, the medium was replaced by a fresh medium and the cells were pretreated with 10 μM polyphenols for 2 h followed by OA at 1.5 mM for 24 h. After 24 h, cells were washed twice with PBS and 1 mL of TRIzol was added to each well for 2 min to lyse the cells. Then, cell lysates were transferred to a clean RNase-free 1.5-mL Eppendorf tube and 200 μL chloroform was added to each sample lysate with vigorous shaking for 10 s. After 3-min incubation at room temperature, the lysates were vortexed again and centrifuged at 12,000 × g for 10 min at 4°C. In the next step, the clear aqueous upper layer was carefully separated and transferred to a new Eppendorf tube and 500 μL isopropanol was added to each microtube. After 5-min incubation at room temperature, each sample was applied to a spin column and centrifuged at 8000 × g for 15 s at 4°C. Then, the flow-through was discarded and 350 μL RW1 buffer (provided with the kit) was added to each sample and centrifuged again at 8000 × g for 15 s and the flow-through discarded. In the next step for removing any DNA contamination, the samples were treated with 80 μL DNase (Invitrogen) and incubated at room temperature for 15 min. Then, the samples were washed with 350 μL of RW1 buffer and centrifuged at 8000 × g for 15 s at 4°C. After discarding the flow-through, the samples were washed with RPE buffer (provided with the kit), spin-dried for 1 min, and the spin columns were transferred to a fresh RNase-free Eppendorf tube. Finally, 50 μL nuclease-free water (Invitrogen) was added to each spin column, which was then centrifuged at 8000 × g for 1 min and the flow-through containing purified total RNA was stored at −80°C for later analyses.

2.6.2 Synthesis of complementary DNA

Frozen RNA samples were thawed and RNA concentration and purity was measured using a Nano Drop spectrophotometer (BioRad) and the A260/A280 absorbance ratio. The A260/A280 absorbance ratio for all extracted RNAs was between 2 and 2.1 showing that total RNA was highly pure (within the acceptable ratio of 1.8–2.1). Each 1 × 10^6 HepG2 cells in our experiment yielded approximately 18 μg total RNA. Using a VILO cDNA synthesis kit (Invitrogen) and a Thermocycler (BioRad), reverse transcription of 2 μg total RNA to complementary DNA (cDNA) was performed in 20 μL reactions. For cDNA synthesis, thermal cycling was adjusted as follows: one cycle at 25°C for 10 min, one cycle at 42°C for 60 min (optimal for synthesis of cDNA), and one cycle at 85°C for 5 min (to inactivate DNA polymerase). cDNA samples were kept in a −80°C freezer and were diluted 20 times before use according to the VILO cDNA synthesis kit protocol to remove PCR inhibitory effects of reverse transcription. All primers were provided by Integrated DNA Technologies (IDT, Canada). Table 1 shows primer sequences used in this study.

2.6.3 PCR

The PCR was performed using a PCR system (ABI 7300; Applied Biosystem) using Power SYBR Green real-time PCR master mix (Life Technologies) in 20 μL reactions, according to the manufacturer’s protocol. Thermal cycling used for PCR was as follows: One cycle at 95°C for 5 min (for enzyme activation), followed by 40 cycles at 95°C for 15 s and 60°C for 30 s. Melting curves of all PCR products were evaluated to confirm the amplification and primers quality. A comparative method (2−ΔΔCT) was used to analyze the relative expression of genes of interest using GAPDH as a reference gene. All results were normalized to GAPDH. Each experiment was repeated in duplicate with three replicate wells per experiment.

2.7 Statistical analysis

Results were expressed as mean values with their SEM for the number of experiments indicated. Results were analyzed using SPSS 22 (IBM, USA). One-way ANOVA was used initially, and when there was any significance detected, Dunnett’s post hoc test was used to compare the mean of each group with control or normal groups. The level of significance was p < 0.05. Correlation was determined by Pearson’s R. The normality of variables was confirmed with the Kolmogorov–Smirnov test.
Table 1. The sequences of primers used to measure the expression of genes of interest

<table>
<thead>
<tr>
<th>Gene</th>
<th>Species</th>
<th>Primer sequence (5’–3’)</th>
<th>Cycles (Ct values)</th>
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<tr>
<td>UCP2</td>
<td>Human</td>
<td>Sense: GAA CGG GAC ACCTTT AGA GAA G &lt;br&gt;Antisense: CAG CAA CAA GAC GAG ATA GAG G</td>
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<tr>
<td>Mn-SOD</td>
<td>Human</td>
<td>Sense: GGA GAT GTT ACA GCC CAG ATA G &lt;br&gt;Antisense: CGT TAG GGCTGA GGT TTG T</td>
<td>19</td>
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<td>PGC1α</td>
<td>Human</td>
<td>Sense: TGA ACT GAG GGA CAGTGA TTT C &lt;br&gt;Antisense: CCA AAG GGT AGCTCA GTT TAT C</td>
<td>23</td>
</tr>
<tr>
<td>NRF1</td>
<td>Human</td>
<td>Sense: GTATCT CAC CCT CCA AAC CTA AC &lt;br&gt;Antisense: CCA GGATCA TGC TCT TGT ACT T</td>
<td>24</td>
</tr>
<tr>
<td>TFAM</td>
<td>Human</td>
<td>Sense: ATA GGC ACA GGA AAC CAGTTA G &lt;br&gt;Antisense: GCA GAA GTC CAT GAG CTG AAT A</td>
<td>24</td>
</tr>
<tr>
<td>TNFα</td>
<td>Human</td>
<td>Sense: GGA TGG ATG GAG GTG AAA GTA G &lt;br&gt;Antisense: TGA TCC TGA AGA GGA GAG AGA A</td>
<td>33</td>
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<tr>
<td>NDUFS8</td>
<td>Human</td>
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<tr>
<td>SDHb</td>
<td>Human</td>
<td>Sense: Cat TCT CTG TGC CTG CTG TA &lt;br&gt;Antisense: GGA GTC AAT CAT CCA GCG ATA G</td>
<td>23</td>
</tr>
<tr>
<td>UQCRC1</td>
<td>Human</td>
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<tr>
<td>COX6B1</td>
<td>Human</td>
<td>Sense: CCA ACC AGA ACC AGA CTA GAA A &lt;br&gt;Antisense: GTA CCATTC GCA CAC AGA GAT A</td>
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<tr>
<td>ATP5G1</td>
<td>Human</td>
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<tr>
<td>ATP5F1</td>
<td>Human</td>
<td>Sense: GTC CCT TTG TTG CAG ACT TTG &lt;br&gt;Antisense: CCT GTT GTG ACT TCT CCG TAT C</td>
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<td>GAPDH</td>
<td>Human</td>
<td>Sense: ATG GGT GTG AAC CAT GAG AAG &lt;br&gt;Antisense: GAG TCC TTC CAC GAT ACC AAA G</td>
<td>17</td>
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3 Results

3.1 Effect of OA and polyphenols on ROS generation in HepG2 cells

The treatment of HepG2 cells with 1.5 mM OA for 24 h significantly increased ROS generation by 127% (Fig. 1a). All polyphenols at different concentrations except delphinidin (5 and 10 μM) significantly decreased ROS generation. Compared to the OA alone condition, cyanidin-3-glucoside (kuromanin) was the strongest polyphenol and at 10 μM decreased ROS by approximately 77%. Delphinidin inhibited significantly at 1 μM, but at 5 and 10 μM was unable to significantly prevent ROS generation.

Figure 1b shows the effect of quercetin and cyanidin, and their phenolic breakdown/digestion products on ROS generation. The treatment of HepG2 cells with 750 μM OA increased ROS generation by 73%, while pretreatment with 10 μM quercetin, cyanidin, and their phenolic products such as caffeic acid, protocatechuic acid, and 2,4,6-trihydroxybenzaldehyde prevented ROS generation between 71 and 105%.

3.2 Effects of OA and polyphenols on mitochondrial content

Evaluating mitochondrial number by MitoTracker Green shows that OA significantly decreased mitochondrial content in hepatocytes by 21% and some of the polyphenols protected against this reduction (Fig. 1c and d). The strongest compound was berberine followed by quercetin and resveratrol, which reversed OA-induced inhibition of mitochondrial content to levels higher than control cells.
3.3 Effect of OA and polyphenols on the expression of genes of interest

Since our present study showed that polyphenols at 10 μM have the strongest protective effects against OA-induced ROS generation, the effect of polyphenols at this dose was studied on the expression of genes involved in oxidative stress, inflammation, and mitochondrial biogenesis.

3.3.1 Polyphenols reversed an OA-induced decrease in expression of uncoupling protein 2

Figure 2a shows relative uncoupling protein 2 (UCP2) mRNA expression after pretreatment with different polyphenols (10 μM) for 2 h and OA (1.5 mM) for 24 h. OA significantly inhibited UCP2 mRNA expression by 37%. Catechin, quercetin, cyanidin, and kuromanin significantly reversed OA-induced inhibition of UCP2 expression by more than 100%. Although resveratrol and berberine appeared to rescue UCP2 expression by 76 and 78%, this induction did not reach significance (p = 0.18 and P = 0.15, respectively).

3.3.2 Some polyphenols increased the expression of mitochondrial manganese superoxide dismutase

OA alone had no significant effect on manganese superoxide dismutase (Mn-SOD) expression (Fig. 2b). Catechin, quercetin, and resveratrol significantly increased Mn-SOD expression by 40, 39, and 35%, respectively, compared to OA alone. The anthocyanins and berberine had no significant effect on Mn-SOD expression.

3.3.3 Polyphenols except anthocyanins prevented OA-induced expression of TNF-α

OA elevated mRNA expression of TNF-α by 147% while polyphenols could prevent this induction (Fig. 2c). Resveratrol, berberine, catechin, and quercetin inhibited OA-induced TNF-α expression by 98, 94, 75, and 60%, respectively. Anthocyanins (cyanidin and kuromanin) did not give any inhibition.

3.3.4 Polyphenols except anthocyanins induced the expression of genes involved in mitochondrial biogenesis (PGC1α, NRF1, and TFAM)

OA alone significantly inhibited peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC1α) mRNA expression by 21%, while berberine, resveratrol, quercetin, and catechin induced its expression by 84, 63, 42, and 37% compared to control cells, respectively (Fig. 3a). Moreover, OA significantly decreased NRF1 (where NRF is nuclear respiratory factor) mRNA expression by 26% and different polyphenols such as catechin, berberine, and quercetin significantly increased NRF1 expression by 66, 43, and 40%, respectively, compared to OA alone. Additionally, while treatment with oleic alone had no significant effect on mitochondrial transcription factor A (TFAM) mRNA expression,
berberine, catechin, and resveratrol significantly induced TFAM expression by 77%, 71%, and 71%, respectively, compared to OA alone. Although quercetin appeared to increase TFAM expression by 54%, it was not significantly different from OA alone \((p = 0.18)\). Anthocyanins such as cyanidin and kuromanin were unique in having no significant effect on expression of genes involved in mitochondrial biogenesis.

### 3.3.5 OA and polyphenols influence the expression of nuclear DNA encoded mitochondrial respiratory complex subunits

The effect of OA and polyphenols on mRNA expression of nuclear DNA encoded mitochondrial complex subunits is shown in Fig. 3b. While OA alone significantly decreased mRNA expression of mitochondrial respiratory complex I subunit (NADH dehydrogenase (ubiquinone) Fe-S protein 8 \((\text{NDUFS8})\)) by 49% compared to untreated cells, all polyphenols except cyanidin protected against this inhibition and in most cases increased the expression to the level more than untreated cells. OA alone had no significant effect on mRNA expression of \(\text{SDHb}\) (complex II subunit), mitochondrial complex III (ubiquinol-cytochrome c reductase core protein I \((\text{UQCRC1})\)), or IV \((\text{COX6B1})\) mRNA expression. Among polyphenols, only berberine increased expression of \(\text{SDHb}\) (by 81%), while the treatment with catechin, resveratrol, and cyanidin significantly increased \(\text{UQCRC1}\) expression (by 58, 46, and 45%, respectively), and berberine and catechin were able to significantly increase \(\text{COX6B1}\) expression (by 34 and 31%, respectively). While OA alone significantly inhibited the expression of complex V subunit \((\text{ATP5G1})\) by 29%, no polyphenol protected against this inhibition (Fig. 3b).

### 3.4 Gene expression correlations

We found a significant positive correlation between \(\text{PGC1a}\) and \(\text{Mn-SOD}\) expression \((r = 0.4, p = 0.005)\) and a significant inverse correlation between \(\text{PGC1a}\) and \(\text{TNF-\alpha}\) \((r = -0.54, p < 0.001)\), which are shown in Fig. 4. Also, there was a significant inverse correlation between \(\text{UCP2}\) gene expression and ROS generation \((r = -0.42, p = 0.003)\). A significant inverse correlation was also found between mitochondrial respiratory complex I \(\text{NDUFS8}\) and III \(\text{UQCRC1}\) subunits and \(\text{TNF-\alpha}\) mRNA expression \((r = -0.44, p = 0.003, \text{and } r = -0.45 \text{ and } p = 0.002, \text{respectively})\).

### 4 Discussion

Oxidative stress and inflammation have been demonstrated as the second hits, which accelerate the progression of simple steatosis to NAFLD, and dietary polyphenols of polyphenol-rich foods have shown promise in protecting from NAFLD. Therefore, we compared the preventive effect of different classes of polyphenols on these hits in an in vitro model of steatosis to explore the effects on molecular mechanisms underlying NAFLD.

In this model, 1.5 mM OA significantly induced ROS generation in HepG2 cells by 127%. Zhang et al. [23] previously showed that OA at 1 mM could increase ROS generation in HepG2 cells by 80%. The higher concentration of OA (1.5 mM) in our study gave a slightly greater increase in ROS generation (127%) compared to this previous study. In line with this finding, Liu et al. [30] observed that 1.5 mM OA, but not lower doses increased ROS levels in HepG2 cells, as well as release of ALT and AST from the cells. This dose
is slightly above the mean plasma concentration of OA observed in healthy young humans of 1.3 mM [31]. Consistent with the link between oxidative stress and inflammation, we also found that OA increased expression of the inflammatory cytokine TNF-α. Together the results suggest that this model can be a useful in vitro model for studying molecular mechanisms underlying NAFLD because of increased ROS generation and production of inflammatory cytokines.

All of the tested polyphenols strongly inhibited OA-induced ROS generation. One reason that they decreased ROS generation may have been direct antioxidant activities [32]. However, we also found increased expression of the antioxidant enzyme Mn-SOD by some polyphenols (resveratrol, catechin, and quercetin) and ROS-decreasing enzyme UCP2 in hepatocytes, which may further explain the ROS-reducing effect of these compounds. Together these mechanisms resulted in decreased intracellular ROS, which may be one of the main second hit mechanisms by which polyphenols inhibit progression of NAFLD to NASH. In NAFLD, excess fatty acids are oxidized leading to increased ROS generation. This increased ROS can result in mitochondrial dysfunction that increases hepatic steatosis in the liver, and this vicious circle finally leads to cirrhosis and hepatocellular carcinoma [33]. Therefore, our results show that by suppressing ROS generation in hepatocytes, polyphenols may have the potential to break this vicious circle and prevent mitochondrial dysfunction.

Delphinidin was unique in showing lower protection with increasing concentration from 1 to 10 μM. This effect may be due to higher concentrations producing more prooxidant activity [34]. Due to having three hydroxyl groups in the B-ring, delphinidin would have a lower reduction potential than the other flavonoids and be more easily oxidized by molecular oxygen (catalyzed by metals in the cell culture medium) to produce ROS.

Another study [23] was in agreement with our results showing polyphenol inhibition of ROS generation in HepG2 hepatocytes. In this study, different flavonoids had different potencies in inhibiting OA-induced ROS generation and triglyceride accumulation. The potency was not clearly related to the extracellular radical-reducing potential of the flavonoid, suggesting additional mechanisms. This previous study included two of the flavonoids in the current study (catechin and quercetin), but not anthocyanins or other polyphenols such as resveratrol described herein.

We also found that the dietary phenolic caffeic acid and the quercetin and cyanidin breakdown/digestion products protocatechuic acid and 2,4,6-trihydroxybenzaldehyde gave similar protection against ROS generation (Fig. 1b). During digestion, flavonoids are partially broken down to phenolic products by gut bacteria, and these products have been suggested to contribute to beneficial effects in vivo [35–37]. This suggests that the protective effects of dietary polyphenols such as quercetin and cyanidin against ROS generation can be due to both the parent polyphenols and their breakdown/digestion products.

Another second hit that we investigated in HepG2 cells was inflammation. The results showed strong OA-induced TNF-α expression and inhibition by the polyphenols (except cyanidin and kuromanin). An accumulating body of evidence shows that NAFLD-associated ROS and lipid peroxidation aldehydes (malondialdehyde and 4-hydroxynonenal) result in increased release of cytokines such as TNF-α and Fas ligand, which play a major role in inflammation, cell death, and fibrosis [17, 18]. Increased release of TNF-α by hepatocytes, adipocytes, and Kupffer cells (residing macrophages in liver) results through activation of nuclear factor kappa b by ROS (reviewed in [39]) and may contribute to the severity of mitochondrial dysfunction seen in NASH patients [38]. Elevated levels of hepatic TNF-α expression was reported in obese patients with NAFLD, and the level of TNF-α was correlated with advanced hepatic fibrosis in these patients [40].

Other studies in experimental models of NAFLD have shown benefits of polyphenols against inflammation. In line with our result, treating HepG2 cells with OA was previously...
Figure 4. Correlations between different parameters of interest in our study: (a) Positive correlation between PGC1α and Mn-SOD mRNA expression. (b) Inverse correlation between PGC1α and TNFα mRNA expression representing increased mitochondrial biogenesis is correlated with decreased inflammation. (c) Inverse correlation between UCP2 mRNA expression and ROS representing higher UCP2 expression is correlated with decreased ROS generation in hepatocytes. (d and e) Inverse correlation between mitochondrial respiratory complex I NDUF8 and III UQRC1 subunits and TNFα mRNA expression, which shows decreased level of inflammation along with improved function of mitochondrial complexes.

shown to induce mRNA expression of TNF-α by more than 2.5-fold, and quercetin at 10 μM was effective in its inhibition [41]. In studies on animals fed a high-fat diet, dietary quercetin inhibited hepatic steatosis and decreased liver and plasma levels of the pro-inflammatory cytokines TNF-α and IL-6 [11, 21].

Because lower mitochondrial biogenesis may have a major role in NAFLD and progression to NASH [9], we investigated the effects of OA and polyphenols on mitochondrial expression in the HepG2 cell model of steatosis. In the present study, we observed decreased mitochondrial biogenesis in HepG2 cells exposed to OA, shown by decreased MitoTracker fluorescence, decreased expression of the mitochondrial biogenesis transcription factors PGC1α and NRF1, and decreased expression of respiratory complex I and V subunits NDUF8 and ATP5G1. An accumulating body of evidence reports that mitochondrial number and function are decreased in subjects with obesity and diabetes [42, 43], which are the major contributors of NAFLD/NASH. In line with our results, Koliaki et al. [44] also reported decreased hepatic mRNA expression of PGC1α, NRF1, and TFAM in patients with NAFLD and NASH. A reduced number of hepatic and muscle healthy mitochondria result in reduced β-oxidation of fatty acids while producing more ROS.

Other studies support that a high-fat diet decreases expression of mitochondrial respiratory complex proteins. In one study [45], feeding an isocaloric high-fat diet decreased expression of nuclear-encoded mitochondrial complex subunits in skeletal muscle of rodents and humans. In a previous study on NASH in mice [46], the authors found that the activity of all of the mitochondrial respiratory complexes in liver was reduced by 50–60% by a high-fat diet and this decline was particularly due to decreased gene expression of mitochondrial DNA encoded subunits. Those authors also found that due to the protection of vulnerable mitochondrial DNA against ROS, administration of antioxidants such as MnTBAP (a superoxide dismutase mimetic) and uric acid was effective to reverse these effects induced by high-fat diet.

In the present study, we also show that most polyphenols increased mitochondrial content along with increased expression of genes involved in mitochondrial biogenesis (PGC1α, NRF1, and TFAM) to levels more than in control cells. The anthocyanins (cyanidin and kuromanin), however, had no effect on mitochondrial biogenesis. This could be because anthocyanins, unlike other polyphenols such as resveratrol, reportedly have no direct effect on SIRT1 activity [47], which is shown to be one of the most important regulators of mitochondrial biogenesis by activating AMP-activated protein kinase and PGC1α [48]. In agreement with our findings, resveratrol has been found to reverse the inhibition of mitochondrial biogenesis in liver of mice fed a high-calorie high-fat diet [19]. Gomes et al., [49] also observed that berberine could protect against high-fat diet-induced inhibition of PGC1α and TFAM gene expression in muscle of rats.

Interestingly, catechin induced the expression of genes involved in mitochondrial biogenesis without induction of mitochondrial mass. One explanation may be that catechin increased mitochondrial differentiation more than mitochondrial replication. Mitochondrial differentiation affects preexisting mitochondria and improves mitochondrial function by expanding mitochondrial inner membrane with more cristae and increasing expression of mitochondrial proteins such as respiratory complex subunits [50, 51]. Although
having no effect on mitochondrial mass, catechin induced the expression of mitochondrial respiratory complex I, III, and IV subunits. Accordingly, catechin appeared to have a more distinct effect on mitochondrial differentiation.

Defective mitochondrial oxidative phosphorylation is reported in the liver of subjects with NASH [52], as well as in several models of NAFLD, including HepG2 hepatocytes treated with palmitate [53], obese ob/ob mice with NAFLD [54], and mice fed a high-fat diet [46]. It was suggested that a decrease in mitochondrial biogenesis (decreased number of healthy and functional mitochondria) and impaired expression and assembly of mitochondrial respiratory complex subunits may result in increased ROS production, which is one of the main contributors to NAFLD [43]. Therefore, improving mitochondrial function and electron flow in the mitochondrial electron transport chain (ETC) may be of vital importance to prevent ROS generation in NAFLD. We found that OA inhibited complex I and V subunits (NDUFS8 and ATP5G1) expression. Importantly, complex I is the main entry point of electrons to mitochondrial respiratory chain. Most polyphenols protected against this inhibition and in many cases increased the expression of NDUFS8 to significantly more than control cells. Our present study suggests that in opposition to increased load of NADH and electrons to enter the mitochondrial ETC in fatty liver disease, polyphenols induce the expression of subunits of mitochondrial complexes to increase flow of electrons, which may lead to increased fat oxidation and decreased ROS generation.

We found a significant positive correlation between PGC1α and Mn-SOD mRNA expression, suggesting that this effect could be due to increased mitochondrial biogenesis and number by PGC1α. Additionally, PGC1α has been shown to have a major role in regulating ROS removal by inducing antioxidant defense such as glutathione peroxidase, superoxide dismutase, and catalase [55, 56].

The mitochondrial enzyme UCP2 is another enzyme implicated in mitochondrial ROS generation and fatty acid β-oxidation. In the present study, we found decreased expression of UCP2 by OA, while most polyphenols prevented this decrease. UCP2 is localized in the inner mitochondrial membrane and uncouples oxidative phosphorylation from ATP production [57], resulting in decreased electrochemical potential (ΔΨ) and decreased production of mitochondrial ROS [58, 59]. Studies report that even small changes in ΔΨ can have an order of magnitude effect in decreasing mitochondrial ROS generation [60]. In agreement with this connection, we found an inverse correlation between UCP2 expression and ROS generation.

In line with our results with polyphenols, other evidence suggests that dietary polyphenols (such as resveratrol and the flavonoid naringenin) can induce the hepatic expression of PGC1α and NRF1. However, polyphenols except anthocyanins induced mitochondrial biogenesis in HepG2 cells leading to overexpression of antioxidant enzyme Mn-SOD, which further suppresses ROS production. Reduced ROS production by polyphenols in turn decreases TNFα expression and prevents inflammation. Polyphenols also protected against oleic acid induced decrease in mitochondrial complex I subunit expression and increased the expression of complex III and IV subunits, which may help flow of electrons. This flow of electrons most likely inhibits leakage of electrons from complex I and III resulting in decreased ROS production. Polyphenols also reversed oleic acid induced decrease in UCP2 expression, which by facilitating import of H+ to mitochondrial matrix suppresses ROS production and may improve flow of electrons in electron transport chain.

Figure 5. Schematic representing how polyphenols inhibit ROS production, inflammation, and improve mitochondrial biogenesis. Oleic acid inhibits mitochondrial biogenesis by blunting expression of PGC1α and NRF1. However, polyphenols except anthocyanins induced mitochondrial biogenesis in HepG2 cells leading to overexpression of antioxidant enzyme Mn-SOD, which further suppresses ROS production. Reduced ROS production by polyphenols in turn decreases TNFα expression and prevents inflammation. Polyphenols also protected against oleic acid induced decrease in mitochondrial complex I subunit expression and increased the expression of complex III and IV subunits, which may help flow of electrons. This flow of electrons most likely inhibits leakage of electrons from complex I and III resulting in decreased ROS production. Polyphenols also reversed oleic acid induced decrease in UCP2 expression, which by facilitating import of H+ to mitochondrial matrix suppresses ROS production and may improve flow of electrons in electron transport chain.
these complexes, which finally inhibits ROS production and inflammation.

In conclusion, dietary polyphenols of different classes were similarly effective in decreasing OA-induced ROS generation in human hepatocytes. As part of the mechanism, all polyphenols except anthocyanins increased mitochondrial biogenesis and the expression of mitochondrial respiratory complex subunits. Figure 5 schematically represents how polyphenols may inhibit ROS production, decrease inflammation, and improve mitochondrial biogenesis and function in HepG2 cells exposed to OA.

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5 References


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