Hydroxytyrosol improves mitochondrial function and reduces oxidative stress in the brain of \textit{db/db} mice: role of AMP-activated protein kinase activation

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Abstract

Hydroxytyrosol (HT) is a major polyphenolic compound found in olive oil with reported anti-cancer and anti-inflammatory activities. However, the neuroprotective effect of HT on type 2 diabetes remains unknown. In the present study, \textit{db/db} mice and SH-SY-5Y neuroblastoma cells were used to evaluate the neuroprotective effects of HT. After 8 weeks of HT administration at doses of 10 and 50 mg/kg, expression levels of the mitochondrial respiratory chain complexes I/II/IV and the activity of complex I were significantly elevated in the brain of \textit{db/db} mice. Likewise, targets of the antioxidative transcription factor nuclear factor erythroid 2 related factor 2 including p62 (sequestosome-1), haeme oxygenase 1 (HO-1), and superoxide dismutases 1 and 2 increased, and protein oxidation significantly decreased. HT treatment was also found to activate AMP-activated protein kinase (AMPK), sirtuin 1 and PPAR\textsubscript{g} coactivator-1z, which constitute an energy-sensing protein network known to regulate mitochondrial function and oxidative stress responses. Meanwhile, neuronal survival indicated by neuron marker expression levels including activity-regulated cytoskeleton-associated protein, \textit{N}-methyl-D-aspartate receptor 1; Nrf2, nuclear transcription factor erythroid 2 related factor-2; p-AMPK, phospho-AMPK, PGC-1, PPAR\textsubscript{g} coactivator-1, ROS, reactive oxygen species; Sirt1, sirtuin 1; SOD, superoxide dismutase; T2DM, type 2 diabetes mellitus; TBST, Tris-buffered saline Tween 20.

Key words: Hydroxytyrosol: \textit{db/db} Mice: Mitochondrial function: Oxidative stress: AMP-activated protein kinase pathway: Reactive oxygen species

The escalating epidemic of the metabolic syndromes, including obesity and diabetes, represents one of the most pressing and costly medical challenges in public health of the twenty-first century. A recent study has reported that 336 million people had diabetes in 2011, and this number is expected to rise to 552 million by 2030\textsuperscript{1}. Diabetes is recognized by hyperglycaemia and glucose intolerance due to insulin deficiency, impaired insulin sensitivity or both. Type 2 diabetes mellitus (T2DM) is predominant and accounts for 90\% of patients with diabetes\textsuperscript{2}. It is well established that diabetes has been associated with slowly progressing brain damage that impairs cognitive function\textsuperscript{3–5}. Although there is some evidence for a relationship between T2DM and brain damage, the mechanisms driving it remain unknown.

Clinical studies have indicated that cognitive dysfunction is correlated with brain atrophy in patients with diabetes\textsuperscript{3,6,7}. Kumar et al.\textsuperscript{8} reported smaller total brain grey matter volumes in patients with diabetes, and the decrease is more pronounced in the cortical grey matter of the temporal lobe\textsuperscript{9}. Despite the limited knowledge on the underlying mechanisms for cognitive dysfunction during the progression of T2DM, mitochondrial dysfunction and oxidative stress have been suggested as major contributors\textsuperscript{10–12}. A previous study has indicated that high glucose concentration, a major pathological characteristic of diabetes, could induce...
overproduction of superoxide from mitochondrial electron transport chain, which is considered the first and key event involved in the pathogenesis of diabetic and subsequent complications(13). Overexpression of Mn superoxide dismutase (SOD2) protects neurons against hyperglycaemic injury in db/db diabetic mice(14). It has also been reported that high glucose concentrations could enhance the formation of advanced glycation end products, which can induce reactive oxygen species (ROS) production from the mitochondria and promote oxidative damage to the heart and brain(15,16). Given the importance of the mitochondria as a source of energy and potential link in the pathogenesis between diabetes and neurodegenerative diseases such as Alzheimer’s disease(17). While numerous mechanisms regulating mitochondrial function have been delineated, including mitochondrial biogenesis, dynamics, modification and mitophagy, the clear mechanisms accounting for mitochondrial dysfunction during T2DM-associated brain damage remain to be elucidated.

Hydroxytyrosol (HT), a natural polyphenol from virgin olive oil, is considered to be one of the most effective antioxidants. Consumption of HT has certain health benefits, and the responsible mechanisms for these effects have been mainly attributed to its ability to scavenge ROS and enhance endogenous antioxidant systems(18,19). In our previous studies, we have found that HT could protect retinal epithelial pigment cells and adipocytes against oxidative damage through activating the nuclear transcription factor erythroid 2p45-related factor-2 (Nrf2)/Kelch-like ECH-associated protein 1 pathway(20–22) and stimulate mitochondrial biogenesis through PPARγ coactivator-1 (PGC-1α) activation(23,24). These studies have suggested that HT possesses the ability to reduce oxidative stress and improve mitochondrial function. However, the beneficial effects of HT on diabetic brain damage remain unknown. Thus, because HT was reported to efficiently cross the blood–brain barrier(24), it was used in the present study to explore the protective effects of HT on diabetic brain damage.

**Experimental methods**

**Chemicals**

An antibody against β-actin was obtained from Sigma. Antibodies against complexes I (NADH dehydrogenase (ubiquinone) Fe-S protein 3; NDUF3), II (subunit 30 kDa), III (subunit core 2), IV (subunit I) and V (subunit α) were obtained from Invitrogen. Antibodies against AMP-activated protein kinase (AMPK), phospho-AMPK (p-AMPK) and PGC-1 were obtained from Cell Signaling Technology. Antibodies against AMP-activated protein kinase (AMPK), phospho-AMPK (p-AMPK) and PGC-1 were obtained from Invitrogen. Antibodies against AMP-activated protein kinase (AMPK), phospho-AMPK (p-AMPK) and PGC-1 were obtained from Invitrogen. Antibodies against AMP-activated protein kinase (AMPK), phospho-AMPK (p-AMPK) and PGC-1 were obtained from Invitrogen. Antibodies against AMP-activated protein kinase (AMPK), phospho-AMPK (p-AMPK) and PGC-1 were obtained from Invitrogen. Antibodies against AMP-activated protein kinase (AMPK), phospho-AMPK (p-AMPK) and PGC-1 were obtained from Invitrogen. Antibodies against AMP-activated protein kinase (AMPK), phospho-AMPK (p-AMPK) and PGC-1 were obtained from Invitrogen. Antibodies against AMP-activated protein kinase (AMPK), phospho-AMPK (p-AMPK) and PGC-1 were obtained from Invitrogen. Antibodies against AMP-activated protein kinase (AMPK), phospho-AMPK (p-AMPK) and PGC-1 were obtained from Invitrogen.

**Real-time PCR**

Total RNA was extracted from cells using TRIzol reagent according to the manufacturer’s instructions. Reverse transcription from RNA to complementary DNA was performed using the PrimeScript RT-PCR Kit (TaKaRa) followed by semiquantitative real-time PCR using gene-specific primers. The following primers were used for the RT-PCR analysis: activity-regulated cytoskeleton-associated protein (Arc), GGTAAAGTGCCCCAGCTGAGATG (forward) and CCACGTGTAACCCCTTTC (reverse); N-methyl-D-aspartate receptor 1 (NMDAR1), GCCCAACCCCATACAGATG (forward) and GGCGGGTCCTAATGAGATG (reverse); nerve growth factor (NGF), TTGCGAGGC-GCGCTT (forward) and TGGCTGTACGGCGATCAA

from Invitrogen, and the immunoprecipitation lysis buffer was from Beyotime. HT was purchased from Xi’an APP-Chem Bio(Tech) Company Limited.

**Animals and treatments**

Male db/db mice aged 4-week-old from a C57BL/6j genetic background were purchased from SLAC Laboratory Animals Company Limited. After 1 week of acclimatization, mice were randomly divided into the following three groups: db/db mice; db/db mice with a daily oral administration of low-dose HT (10 mg/kg per d); db/db mice with a daily oral administration of high-dose HT (50 mg/kg per d). After 8 weeks of feeding, mice were fasted overnight and killed. All animals were housed in a temperature-controlled (25–27°C) and humidity-controlled (60%) animal room and maintained on a 12 h light–12 h dark cycle (light from 08.00 to 20.00 hours) with food and water provided during the experiments. All the procedures were performed in accordance with the United States Public Health Services Guide for the Care and Use of Laboratory Animals, and all efforts were made to minimise the suffering and the number of animals used in the present study.

**Western blotting**

Cortical brain tissue was lysed with Western and immunoprecipitation lysis buffer. Lysates were centrifuged at 13 000g for 10 min at 4°C. Supernatant protein concentrations were determined with a BCA protein assay kit (Pierce). Equal amounts of protein per sample (20 μg) were subjected to 10 % (w/v) SDS–PAGE; proteins were then transferred to nitrocellulose membranes and blocked with a 5 % (w/v) non-fat milk/TBST (Tris-buffereu saline Tween 20) for 1 h at room temperature. Membranes were incubated with primary antibodies against β-actin (1:5000), mitochondrial complex I–V (1:3000), AMPK (1:1000), phospho-AMPK (1:1000), PGC-1 (1:1000), Sirt1 (1:1000), p62 (1:1000), HO-1 (1:1000), SOD1 (1:1000) and SOD2 (1:1000) in 5 % (w/v) non-fat milk/TBST at 4°C overnight. Membranes were washed with TBST three times and were then incubated with anti-rabbit or anti-mouse antibodies at room temperature for 1 h. Chemiluminescence detection was performed by an ECL Western blotting detection kit (Pierce) and quantified by scanning densitometry.
Assays for mitochondrial complex activities

Mitochondria were isolated from mouse cortical brain tissue, and mitochondrial protein concentrations were determined using the BCA protein assay kit (Pierce). NADH–ubiquinone reductase (complex I) activities were measured spectrometrically using conventional assays, as described previously (25).

Cell viability assay

SH-SY-5Y cells were seeded in ninety-six-well plates at a density of 4 x 10⁴ cells per well for 24 h. These cells were then treated with different concentrations of glucose or HT for the indicated time periods. Then, the number of viable cells was determined by addition of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide. Optical densities were read at 550 nm using a microplate spectrophotometer (Multiskan Ascent; Thermo Fisher Scientific, Inc.).

Oxidative status assessment

2',7'-Dichlorodihydrofluorescein diacetate (2',7'-dichlorofluorescin diacetate) is a freely permeable fluorogenic tracer used for the assessment of oxidative status. After treatment, cells were incubated with 10 mM-2',7'-dichlorofluorescein diacetate for 30 min and then washed with PBS three times. Cell lysis was prepared with a lysis solution (10 mM-Tris, 150 mM-NaCl, 0.1 mM-EDTA and 0.5 % Triton X-100, pH 7.5). The supernatant (200 μl) was analysed using a spectrofluorometer with excitation 485 nm and emission 538 nm (Fluoroskan Ascent; Thermo Fisher Scientific, Inc.). An aliquot of supernatant was used in a BCA protein assay to determine the concentration of total protein. ROS levels were expressed as the relative 2',7'-dichlorofluorescein (DCF) fluorescence per μg of protein.

V_O2

Cell VO2 by intact cells, which is a marker for mitochondrial respiration activity, was measured. Basal, oligomycin and FCCP-treated VO2 rates were investigated after high glucose or HT treatment using Seahorse Extracellular Flux Analyzer (Seahorse Bioscience) according to the manufacturer’s instructions.

Statistical analysis

Data are presented as means with their standard errors. The statistical significance of differences among groups were analysed using a one-way ANOVA followed by Dunnett’s multiple comparison test. A P value <0.05 was considered to be statistically significant.

Fig. 1. Effects of hydroxytyrosol (HT) on the expression and activities of mitochondrial complexes in the db/db mouse brain cortex. After 8 weeks of treatment with HT, mice were killed for brain protein and mitochondrial isolation. Protein expression levels of brain mitochondrial complex subunits were determined by the following Western blotting analysis: Western blot image (a), statistical analysis of complex I subunit (b), complex II subunit (c) and complex IV subunit (d) levels. Mitochondrial respiratory chain complex I activity was analysed spectrometrically (e). Expression of each protein was normalised to the β-actin loading control. Values are means, with their standard errors represented by vertical bars (n=8). Mean value was significantly different from that of control group: * P<0.05, ** P<0.01.
**Results**

*Effects of hydroxytyrosol on the expression and activities of mitochondrial complexes in the db/db mouse brain cortex*

The *db/db* mice exhibited obesity and hyperglycaemia relative to normal. Comparing with normal C57BL6 mice, no significant changes were observed on the expression of mitochondrial complexes in the *db/db* mice brain cortex (see online Supplementary Fig. S1). After a 2-month HT supplement in *db/db* mice, the expression of mitochondrial complexes I, II and IV was significantly improved (Fig. 1(a)–(d)), while the expression of complexes III and V was not affected. We also measured the activities of these complexes and found that the activity of complex I was significantly increased after HT treatment compared with the *db/db* control group (Fig. 1(e)), while the activities of other complexes were not changed (data not shown).

*Hydroxytyrosol induced phase II antioxidant systems in the brain of db/db mice*

The induction of phase II enzymes is another effect of HT in our previous *in vitro* studies. In the present study, we found that the phase II antioxidant enzymes, including HO-1 (Fig. 2(a) and (c)), SOD1 (Fig. 2(a) and (d)) and SOD2 (Fig. 2(a) and (e)), were significantly increased by HT treatment. Since SOD2 was found markedly decreased in *db/db* mice compared with C57BL6 control mice (see online Supplementary Fig. S2), the major effect of HT on SOD content in the *db/db* mice was assumed to sufficiently prevent the decrease in SOD2. A previous study has indicated that p62 can activate Nrf2, a key regulator of phase II enzymes, and induce antioxidant proteins and detoxification enzymes. Thus, we measured p62 protein expression and found a significant increase in p62 expression induced after HT treatment (Fig. 2(a) and (b)).

*Hydroxytyrosol inhibited protein oxidation in the brain of db/db mice*

Protein carbonyl content is an indicator of protein peroxidation. As shown in Fig. 3, protein oxidation was effectively decreased by both low and high doses of HT supplements, suggesting that HT may contribute to reduce oxidative stress in the brain of *db/db* mice.

*Hydroxytyrosol activated AMP-activated protein kinase pathway in the brain of db/db mice*

The AMPK/Sirt1/PGC-1 pathway has been suggested as a mechanism for controlling mitochondrial function and Nrf2-regulated antioxidative enzymes. Hence, we detected the AMPK/Sirt1/PGC-1 pathway in the brain of *db/db* mice. As expected, the expression of p-AMPK/AMPK (Fig. 4(a) and (b)), PGC-1 (Fig. 4(c) and (d)) and Sirt1 (Fig. 4(e) and (f)) were significantly increased by HT treatment.
Hydroxytyrosol effect on the brain of db/db mice

Hydroxytyrosol protected against high glucose-induced SH-SY-5Y neuronal cell damage

Hyperglycaemia is a major factor for the complications derived from diabetes. Therefore, a toxicity model induced by high glucose in SH-SY-5Y cells was used to confirm the possible underlying mechanism of HT’s neuroprotective effects in vitro. Similar to a previous report\(^{(31)}\), high levels of glucose (45 and 175 mM) inhibited cell viability in a time-dependent manner within 24 h (Fig. 6(a)). Consistent with previous studies\(^{(32–35)}\), increased DCF fluorescence were observed 3 h after treatment with 45 mM-glucose and plateaued after 6–24 h (Fig. 6(b)). Meanwhile, pre-treatments with 2, 5 and 10\(\mu\)M-HT sufficiently increased cell viability after a 45 mM-glucose challenge (Fig. 6(c)). Although we have previously reported that HT could be efficiently uptake in mice and reached 16\(\mu\)mol/l (2.5\(\mu\)g/ml) at 5 min in serum after 50 mg/kg administration\(^{(25)}\), we also measured HT in culture medium with or without SH-SY-5Y cells, data suggested that HT could efficiently uptake in the cells (see online Supplementary Fig. S4). In addition, we analysed basal and uncoupled mitochondrial VO2 capacity with Seahorse analyzer (Seahorse Bioscience) (see online Supplementary Fig. S5). Normalised data indicated that the basal VO2 capacity, ATP production potential and maximal respiration were all significantly decreased by a 45 mM-glucose treatment, while basal and maximal respiration were efficiently protected by a 10\(\mu\)M-HT pre-treatment (Fig. 6(d)).

Hydroxytyrosol improved neuronal survival of db/db mice

Previous study has reported that neuron loss was observed in the dorsal root ganglia of db/db mice\(^{(30)}\). In the present study, neuron loss was observed in the brain cortex of db/db mice compared with normal C57BL6 control mice evidenced by decreased neuron factors including Arc, NMDAR1 and NGF (see online Supplementary Fig. S3). HT supplements, especially the high dose, significantly increased the mRNA levels of Arc, NMDAR1 and NGF compared with the db/db control mice, suggesting that HT improved neuronal cell survival in the brain of db/db mice (Fig. 5(a)–(c)).

Hydroxytyrosol effect on the brain of db/db mice

**Fig. 3.** Effects of hydroxytyrosol (HT) on protein oxidation in the brain of db/db mice. After 8 weeks of treatment with HT, mice were killed, and the brain tissues were collected. The carbonyl protein content was analysed as an indicator of protein oxidation. (a) Western blotting image and (b) statistical analysis is shown. Expression of protein was normalised to the total protein content loading controls. Values are means, with their standard errors represented by vertical bars \((n=8)\). **Mean value was significantly different from that of control group \((P<0.01)\).

**Fig. 4.** Effects of hydroxytyrosol (HT) on the AMP-activated protein kinase (AMPK) pathway in the brain of db/db mice. After 8 weeks of HT treatment, mice were killed and brain proteins were isolated for Western blotting analysis. The expression of phospho-AMPK (p-AMPK)/AMPK ((a) Western blot image, (b) statistical analysis), PPAR\(\gamma\) coactivator-1 (PGC-1) ((c) Western blot image, (d) statistical analysis) and sirtuin 1 (Sirt1) ((e) Western blot image, (f) statistical analysis) were tested. The expression of each protein was adjusted to AMPK or the \(\beta\)-actin loading control. Values are means, with their standard errors represented by vertical bars \((n=8)\). Mean value was significantly different from that of control group: *\(P<0.05\), **\(P<0.01\).
Hydroxytyrosol prevented high glucose-induced SH-SY-5Y cellular damage through AMP-activated protein kinase activation

Time-dependent treatment of SH-SY-5Y cells with 10 μM HT increased p-AMPK levels at 10 and 30 min, which then gradually decreased, suggesting that HT could rapidly activate the AMPK pathway (Fig. 7(a) and (b)). Meanwhile, HT treatment significantly increased the protein expression of mitochondrial complex IV and HO-1, and the induction effect was abolished by AMPK-specific inhibitor compound C (Fig. 7(c) and (d)). Although HT showed no significant induction on SOD2 protein expression, the inhibition of AMPK sufficiently reduced its content (Fig. 7(c) and (d)), suggesting the major regulatory effect of AMPK pathway on mitochondrial and antioxidative protein expression. In addition, 45 mM-glucose treatment significantly decreased the levels of p-AMPK, while 10 μM-HT pre-treatment effectively restored the levels of p-AMPK.
Hydroxytyrosol effect on the brain of db/db mice

In the present study, HT treatment could significantly improve the expression levels of mitochondrial complexes I/II/IV significantly in the brain and increase the activity of complex I, the major complex in the electron transport chain and oxidative phosphorylation in the db/db mice. In addition, HT treatment increased antioxidative enzymes, such as HO-1, SOD1 and SOD2, and decreased the protein carbonyl content in the brain of db/db mice. Collected data suggested that HT could improve mitochondrial function and reduce oxidative stress in the brain of db/db mice. Therefore, the mitochondrial protective effects and reduction in oxidative stress might be contributing to the increases in neuronal survival.

Previous study has shown 33% neuron loss in the brain of db/db mice at 8-month age compared with the control, which suggested that significant neuron loss may actually happen in the brain of db/db mice. In the present study, we measured the expression of neuron markers including Arc, NMDARI and NGF. Arc has been associated with synaptic potentiation and the long-term consolidation of memory. Arc levels are decreased in high-fat diet-fed animals and Alzheimer's diseased brains. NMDAR, a neurotransmitter receptor, is a key molecule to mediate synaptic plasticity, and the activation of NMDAR is required for Arc function. NGF plays a key role in the development and maintenance of sympathetic and sensory neurons as well as regulation of axonal growth and guidance. Previous studies have reported that NGF could reverse diabetes-related biochemical alterations. In the present study, all the three factors were significantly higher after HT treatment compared with db/db control mice, suggesting that HT has neuroprotective effects.
and may potentially improve neuron survival. Whether this protection is attributed to decreased oxidative stress and the improvement of mitochondrial function or other independent mechanisms warrants further investigation.

AMPK is a key player that regulates energy metabolism and is crucial during the development and treatment of obesity, diabetes and other metabolic disorders. In addition, brain development and function are closely connected to AMPK signalling, which is also involved in neurodegenerative disorders. Impaired AMPK signalling is linked to mitochondrial dysfunction in brain cells and peripheral neuropathy in diabetes. Resveratrol is known to enhance neurite outgrowth and reduce kidney oxidative stress in diabetic rats via activating AMPK signalling. It has also been reported that HT could activate AMPK to reduce intracellular ROS levels in vascular endothelial cells. In this study, p-AMPK and two other regulators of AMPK signalling, Sirt1 and PGC-1, were all significantly increased in the brain of HT-treated db/db mice. To further confirm that the AMPK pathway drives the effects of HT, we chose a high glucose-induced toxicity model in SH-HY-5Y neuroblastoma cells. Similar to previous observations in primary neurons, high glucose significantly induced oxidative stress, mitochondrial dysfunction and decreased cell viability. HT pretreatment efficiently protected mitochondrial function and cell viability, and the addition of compound C, an AMPK inhibitor, abolished these HT effects. Meanwhile, HT induced overexpression of complex IV, one of the mitochondrial electron transport chain and HO-1, one of the antioxidative enzymes was also diminished by compound C. Thus, we suggest that AMPK activation may play major roles in HT-induced mitochondrial biogenesis and phase II enzyme activation, which then protects against high glucose toxicity.

Animal studies have shown that HT can be dose dependently absorbed and excreted in urine, and HT concentrations in rat plasma can reach 1.22 μg/ml in 5 min and 1.91 μg/ml in 10 min after 20 mg/kg HT oral administration, suggesting that the doses of HT used in the present study should be efficiently absorbed and accumulated in vivo. However, the HT content of olive oil varies from 1.55 to 14.42 mg/kg, thus humans with an intake of 50 g olive oil/d would receive 0.7 mg of HT at most. The dose of 10 mg/kg used in the present study could only be achieved in humans through the use of very high dose supplements, typically supplementation studies only achieve a total intake of approximately 50 mg/d. The present study showed that very high doses of HT improved mitochondrial function and induced phase II antioxidative enzymes, which decreased oxidative stress in brain of db/db mice. Furthermore, the activation of AMPK signalling by HT may contribute to its protective effects. However, caution should be exercised in extrapolating these findings to lower doses of HT more typically found in humans. There remains the potential for synergistic effects of HT with other polyphenols in olive oil, which could lead to benefits in a realistic dose range suggesting that HT might be an effective agent for the prevention and treatment of diabetic complications such as brain damage.

Supplementary material
To view supplementary material for this article, please visit http://dx.doi.org/10.1017/S0007114515000884
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The authors declare that they have no conflicts of interest.

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