Dietary Curcumin Ameliorates Aging-Related Cerebrovascular Dysfunction through the AMPK/Uncoupling Protein 2 Pathway

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Key Words
Curcumin • Cerebrovascular dysfunction • Uncoupling protein 2 • 5’-AMP activated protein kinase • Aging

Abstract
Background/Aims: Age-related cerebrovascular dysfunction contributes to stroke, cerebral amyloid angiopathy, cognitive decline and neurodegenerative diseases. One pathogenic mechanism underlying this effect is increased oxidative stress. Up-regulation of mitochondrial uncoupling protein 2 (UCP2) plays a crucial role in regulating reactive oxygen species (ROS) production. Dietary patterns are widely recognized as contributors to cardiovascular and cerebrovascular disease. In this study, we tested the hypothesis that dietary curcumin, which has an antioxidant effect, can improve aging-related cerebrovascular dysfunction via UCP2 up-regulation. Methods: The 24-month-old male rodents used in this study, including male Sprague Dawley (SD) rats and UCP2 knockout (UCP2-/-) and matched wild type mice, were given dietary curcumin (0.2%). The young control rodents were 6-month-old. Rodent cerebral artery vasorelaxation was detected by wire myograph. The AMPK/UCP2 pathway and p-eNOS in cerebrovascular and endothelial cells were observed by immunoblotting. Results: Dietary curcumin administration for one month remarkably restored the impaired cerebrovascular endothelium-dependent vasorelaxation in aging SD rats. In cerebral arteries from aging SD rats and cultured endothelial cells, curcumin promoted eNOS and AMPK phosphorylation, up-regulated UCP2 and reduced ROS production. These effects of curcumin were abolished by either AMPK or UCP2 inhibition. Chronic dietary curcumin significantly reduced ROS production and improved cerebrovascular endothelium-dependent relaxation in aging wild type mice but not in aging UCP2-/- mice. Conclusions: Curcumin improves aging-related cerebrovascular dysfunction via the AMPK/UCP2 pathway.

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Introduction

Aging-related cerebrovascular dysfunction contributes to stroke, cerebral amyloid angiopathy, cognitive decline and neurodegenerative diseases such as Alzheimer's disease [1-3]. Aging can also increase reactive oxygen species (ROS) production, which can reduce nitric oxide (NO) bioavailability and cause cerebrovascular endothelial dysfunction [4, 5].

Uncoupling protein 2 (UCP2) is an inner mitochondrial membrane protein that regulates mitochondrial homeostasis [6-8]. Emerging evidence suggests that UCP2 plays a positive physiological role by regulating fatty acid oxidation, mitochondrial biogenesis, substrate utilization, and ROS elimination [9-11]. Because of its important roles in reducing ROS and regulating mitochondrial function in a diverse range of tissues, UCP2 is also associated with an extended lifespan [6]. Recent studies demonstrated that UCP2-regulated ROS homeostasis protects against ROS-related vascular dysfunction [12, 13]. UCP2 knockout (UCP2^-/-) mice present with increased aortic macrophage infiltration and serious atherosclerotic lesions as well as exacerbated high salt- or high glucose-induced vascular dysfunction [12-14]. UCP2 over-expression preserved endothelial function by inhibiting nicotinamide adenine dinucleotide phosphate (NADPH) activity, thereby reducing ROS production and increasing nitric oxide bioavailability in obese diabetic mice [13]. These studies implicated UCP2 as a promising therapeutic target for treating aging-related cerebrovascular dysfunction.

Curcumin is an extract from the curcuma longa (turmeric) plant rhizome that has been widely used worldwide as a spice and an herbal medicine. Curcumin has many biological activities, including the amelioration of ischemic stroke and anti-oxidant, anti-inflammatory and anti-neurodegenerative properties [15-19]. Recent reports demonstrated that curcumin can modulate activation of the 5'-AMP activated protein kinase (AMPK) signaling pathway, which is associated with aging-related vascular endothelial dysfunction in old mice [20].

In this study, we hypothesized that AMPK activation and curcumin-mediated UCP2 up-regulation would reduce ROS production and prevent aging-related cerebrovascular dysfunction. We provide in vivo and in vitro experimental evidence that curcumin reduced ROS production and increased NO production, thereby rescuing cerebrovascular endothelial dysfunction via the AMPK/UCP2 pathway in aging rodents. Curcumin is thus a useful dietary supplement for treating aging-related cerebrovascular dysfunction.

Materials and Methods

Animal treatments

The investigation conforms to the Guide for the Care and Use of Laboratory Animals that was published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996) and was approved by the Experimental Animal Ethics Committee of Daping Hospital. Aging (24-month-old) male matched UCP2 knockout (UCP2^-/-) and C57BL/6J WT mice were purchased from Jackson Laboratories (Bar Harbor, ME, USA). Aging (24-month-old) male Sprague-Dawley (SD) rats and young (6-month-old) male SD rats were provided by the Experimental Animal Center of Daping Hospital, Third Military Medical University, Chongqing, China. The aging rats and mice were given normal chow (aging control group) or normal chow plus 0.2% curcumin (aging curcumin group) for one month. Young, normal chow-fed rats were used as controls (young control group) [4, 21]. There were 6 animals from each strain for each group. All of the animals were housed under a 12-h/12-h day/night cycle with free access to food and water.

Vasoreactivity measurement by wire myograph

After the rodents were sacrificed, the basilar arteries were removed and dissected in oxygenated ice-cold Krebs solution containing the following (in mmol/L): 119 NaCl, 4.7 KCl, 2.5 CaCl_2, 1 MgCl_2, 25 NaHCO_3, 1.2 KHPO_4 and 11 D-glucose. Changes in the basilar arterial isometric tone were recorded by wire myograph (Danish Myo Technology, Aarhus, Denmark). The arterial segments were stretched to an optimal baseline tension and then equilibrated for one hour before contracting them by exposure to 60 mmol/L KCl and rinsing in Krebs solution. Endothelium-dependent relaxation (EDR) was measured by
assessing concentration-related responses to cumulative acetylcholine (Ach) addition to rings that had been precontracted with 100 nmol/L U46619 for mouse basilar arteries and 10 μmol/L 5-hydroxytryptamine (5-HT) for rat basilar arteries. Some arteries were incubated with the nitric oxide synthase (NOS) inhibitor L-NAME (100 μmol/L, 30 minutes) before measuring EDR. Endothelium-independent relaxation in response to nitroglycerin (NTG) was measured in basilar rings [22, 23].

Rat basilar arterial ring culture
Rodent basilar arterial rings (2 mm in length) were dissected in sterile PBS and incubated in Dulbecco’s modified Eagle’s medium (DMEM, Gibco, Gaithersburg, MD, USA) supplemented with 10% fetal bovine serum (FBS, Gibco) plus 100 IU/mL penicillin and 100 μg/mL streptomycin. Rat basilar arteries were incubated with or without curcumin (10μmol/L) for 12 h and concurrently co-cultured with or without the AMPK inhibitor Compound C (10 μmol/L) or the UCP2 inhibitor Genipin (10 μmol/L).

Cell culture
Pig iliac endothelial cells (PIECs) were grown in DMEM supplemented with 10% FBS and 1% antibiotics. Cultures were maintained at 37°C in a humidified 95% O₂/5% CO₂ atmosphere. Cells were quiesced by incubating 90% confluent cell cultures with serum-free DMEM. Quiescent cells were incubated with or without curcumin (10 μmol/L) for 12 h in the presence or absence of Compound C (10 μmol/L) or Genipin (10 μmol/L).

Dihydroethidium assay
Superoxide production was examined according to a previously described method [24]. Freshly isolated cerebral arteries were embedded in tissue-freezing compound and the specimens were cut into 10-μm sections on cover slips. The artery samples and PIECs were incubated in the dark with dihydroethidium (DHE; Sigma-Aldrich, USA) diluted in Krebs (40 μmol/L) or a DHE-free solution for 45 min at 37°C, followed by three washes in DHE-free Krebs. To quantify DHE fluorescence, the glass slides were placed under an inverted fluorescence microscope (Nikon, Japan). Images were acquired using NIS-Elements 3.2 software (Nikon) and the fluorescence intensity was analyzed.

Evaluation of NO levels
The NO levels in PIECs were assessed by staining with DAF-2 DA (Sigma-Aldrich, USA) in Krebs solution for 45 min at 37°C, followed by three washes with Krebs solution. NO fluorescence was detected and the fluorescence intensity was analyzed as described above [24].

Western blot analysis
Immunoblots of AMPK, p-AMPK, UCP2, eNOS, p-eNOS and GAPDH were prepared as previously described [25, 26]. After incubation with secondary antibodies (ZSGB-BIO, China) at room temperature for 2 h, the proteins were detected with enhanced chemiluminescence and quantified using a Gel Doc 2000 Imager (Bio-Rad, USA). Protein expression was normalized to the internal control, GAPDH. All of the primary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Statistical analysis
Data are presented as the mean ± s.e. The maximum response (Emax) was calculated from individual agonist concentration-response curves using GraphPad Prism 3.0 (GraphPad Software, San Diego, CA). Significant differences in mean values were assessed by Student’s t-test. Two-sided P values <0.05 were considered statistically significant.

Results

Effects of curcumin administration on cerebrovascular endothelial function and ROS production in aging rats.
Ach-induced endothelium-dependent relaxation was impaired in basilar arteries from aging rats compared with those from young rats. Dietary curcumin treatment for one month
remarkably restored the basilar arterial endothelium-dependent relaxation in aging rats (Fig. 1A). This effect was absent in the presence of the eNOS inhibitor L-NAME (Fig. 1B). NTG-induced endothelium-independent relaxation was similar in all of the groups (Fig. 1C).

**Fig. 1.** Effects of curcumin administration on cerebrovascular endothelial function and ROS production in aging rats. A: Representative impaired cerebrovascular endothelium-dependent relaxation was restored by one month of dietary curcumin (Curc, 0.2%) administration (n=6). B: The effects of dietary curcumin on cerebrovascular endothelium-dependent relaxation in aging rats were abolished by pre-incubation with the eNOS inhibitor L-NAME (100μmol/L, 30 min) (n=6). C: NTG-induced endothelium-independent relaxation was similar in all of the groups (n=6). D: One month of dietary curcumin administration had no effect on rat systolic blood pressure (SBP) (n=6). E: Representative examples of one month of dietary curcumin administration on ROS production in aging rat cerebral arteries (n=6). F: Immunoblots demonstrating that dietary curcumin promoted eNOS phosphorylation in aging rat cerebral arteries (n=3). Data are presented as the mean ± s.e. *P<0.05, **P<0.01 versus the young control group (Young); #P<0.05, ##P<0.01 versus the aging control group (Aging). The scale bar represents 50 μm.
Dietary curcumin did not affect the blood pressure in aging SD rats (Fig. 1D). Compared with controls, rats given dietary curcumin demonstrated significantly increased eNOS phosphorylation but reduced ROS production, as evaluated by a dihydroethidium assay (Fig. 1E and F). These results suggest that the impaired endothelial function and increased ROS in aging rat cerebrovascular tissues can be improved by chronic dietary curcumin administration.
Dietary curcumin administration decreased cerebrovascular ROS production and improved endothelium-dependent relaxation in a UCP2-dependent manner in aging mice. Mitochondrial UCP2 regulates ROS production [6]. To explore whether UCP2 participates in curcumin-mediated effects, 24-month-old wild type (WT) and UCP2 knockout (UCP2−/−) mice were evaluated. ROS production was much higher in cerebral arteries from aging UCP2 knockout mice than in those from age-matched WT mice (Fig. 2A). UCP2 knockout mice also demonstrated accelerated cerebrovascular endothelium-dependent relaxation (Fig. 2B and C). One month of dietary curcumin significantly decreased ROS production (Fig. 2A) and improved endothelium-dependent cerebrovascular relaxation in aging WT mice (Fig. 3). Curcumin treatment improved cerebrovascular endothelial dysfunction via the AMPK/UCP2 pathway in aging rats. A: Reduced AMPK phosphorylation was observed in cerebral arteries from aging rats relative to young controls; however, AMPK phosphorylation was substantially rescued by dietary curcumin administration (n=3). B: Immunoblot demonstrating that dietary curcumin treatment promoted UCP2 up-regulation in cerebral arteries from aging rats (n=3). C: Curcumin (Curc, 10 μmol/L, 12 h) treatment improved cerebrovascular endothelium-dependent relaxation in aging rats, but the effects were reversed by pre-incubation with the AMPK inhibitor compound C (Com C, 10 μmol/L) or the UCP2 inhibitor Genipin (Geni, 10 μmol/L), and Com C or Geni pre-incubation alone did not significantly affect vasodilation (n=6). Data are presented as the mean ± s.e. **P<0.01 versus the young control group (Young) for A; **P<0.01 versus the aging control group (Aging) for C; #P<0.05 versus the aging control group (Aging).
2B). These effects of curcumin were not observed in UCP2^-/- mouse cerebral arteries (Fig. 2C). No improvement in cerebrovascular endothelium-dependent relaxation was detected...
in cerebral arteries that were pre-incubated with L-NAME (Fig. 2B and C). There were no significant differences in NTG-induced endothelium-independent vasorelaxation between groups (Fig. 2D and E). Dietary curcumin administration for one month did not affect systolic blood pressure (SBP) levels in aging mice from the wild type (WT) and UCP2−/− groups (Fig. 2F).

Curcumin treatment improved cerebrovascular endothelial dysfunction via the AMPK/UCP2 pathway in aging rats

We next investigated the mechanism underlying the effects of dietary curcumin. AMPK phosphorylation ameliorates age-associated vascular endothelial dysfunction by reducing oxidative stress [21], and dietary curcumin administration counteracts the reduction in rat hippocampal UCP2 levels after brain trauma [18]. Dietary curcumin administration inhibited the reduction in cerebrovascular AMPK phosphorylation in aging rats (Fig. 3A). Treatment of cerebral arteries with curcumin significantly enhanced UCP2 levels compared with cerebral arteries from young and aging rats (Fig. 3B). We then obtained cerebral arteries from aging SD rats and incubated them with 10 μmol/L curcumin for 12 h. Ach-induced cerebrovascular endothelium-dependent relaxation was improved after curcumin incubation (10μmol/L) for 12 h, but these effects were blocked by co-treatment with the AMPK inhibitor Compound C (10 μmol/L) or the UCP2 inhibitor Genipin (10 μmol/L). Compound C or Genipin preincubation did not significantly affect Ach-induced relaxation in basilar arteries from aging rats (Fig. 3C). These results indicate that the curcumin-mediated improvement in cerebrovascular endothelial dysfunction involves activation of the AMPK/UCP2 pathway in aging rats.

Curcumin administration reduces ROS and increases NO production in endothelial cells via the AMPK/UCP2 pathway

To further investigate the mechanisms underlying the curcumin-induced improvement in age-related endothelial dysfunction, we measured the effects of curcumin on endothelial cells. Immunoblot assays demonstrated that curcumin treatment markedly promoted AMPK and eNOS phosphorylation and UCP2 up-regulation. AMPK or UCP2 inhibition, using their respective inhibitors, abolished the effects of curcumin in treated cells compared with controls. However, the UCP2 inhibitor Genipin did not inhibit curcumin-mediated AMPK phosphorylation (Fig. 4A, B and C). In addition, the effects of curcumin on ROS and NO production were evaluated by DHE (red) and DAF-2 DA (green) fluorescent analyses. Curcumin significantly decreased ROS production and increased NO levels compared with controls. However, AMPK inhibitor Compound C or UCP2 inhibitor Genipin administration inhibited the effect of curcumin on ROS and NO production (Fig. 4D and E). These results suggest that curcumin-mediated activation of the AMPK/UCP2 pathway can reduce superoxide anion production and increase NO levels in endothelial cells.

Discussion

This study generated three lines of experimental evidence related to the protective effects of dietary curcumin on cerebrovascular dysfunction. First, impaired cerebrovascular endothelial function and increased ROS in aging rats were improved by chronic dietary curcumin administration via a UCP2-dependent pathway. Second, curcumin administration-mediated UCP2 up-regulation involved AMPK activation in the cerebrovascular endothelium. Third, curcumin-mediated activation of the AMPK/UCP2 pathway antagonized superoxide anion production and prevented NO reduction in endothelial cells.

Strokes primarily occur in elderly people. Preventing cerebrovascular abnormalities in the elderly has important clinical implications for both stroke treatment and stroke prevention [1]. Age-related cerebrovascular dysfunction contributes to ischemic stroke,
intracerebral hemorrhages, microbleeds, cerebral amyloid angiopathy and cognitive decline [1, 2, 5]. ROS levels are important determinants of age-related cerebrovascular endothelial performance including ischemic stroke vulnerability [5, 19]. ROS removal is an effective treatment for cerebrovascular diseases; however, exogenous antioxidant supplements have not had promising effects on ROS-related vascular diseases [27, 28]. Therefore, reducing endogenous ROS activities may be one potential method of improving cerebrovascular function. This study also demonstrated that endothelium-dependent relaxation in basilar arteries was impaired in aging rats. This phenomenon was associated with increased ROS production and reduced eNOS phosphorylation in the cerebrovasculature.

The aging-associated reduction in AMPK activity is an important factor that contributes to reduced mitochondrial function [29-31]. Several studies have demonstrated that increased AMPK activity can extend lifespan [31-35]. Mitochondrial UCP2 plays an important role in aging. The absence of UCP2 shortens the lifespan of wild-type mice, and UCP2 levels positively correlate with postnatal survival of superoxide dismutase-2 mutant animals [6]. In the fruit fly, human UCP2 over-expression in adult neurons decreases oxidative damage and extends lifespan [36]. We also demonstrated that AMPK phosphorylation was lower but that UCP2 levels were slightly higher in the aging rat cerebrovasculature. A previous study demonstrated that increased mitochondrial UCP2 in response to elevated superoxide levels reflects feedback regulation of ROS production in response to chronic oxidative stress [37].

Stroke incidence and poor outcomes after stroke both increase with age. Elderly patients with ischemic stroke often receive less effective treatments [1]. Neuroprotective agents for ischemic stroke have been sought for decades, but none have proved effective in humans because most preclinical studies were performed in young animals. Several epidemiological studies suggest that flavonoid-rich beverages are associated with reduced stroke and dementia risk [15, 38]. Curcumin is a safe, naturally occurring polyphenol from the plant *Curcuma longa*. It is found in the spice turmeric and has long been used in traditional medicine [15, 16, 18, 19]. Curcumin has anti-oxidant and anti-inflammatory properties [15-19]; however, the effects of curcumin on aging-related cerebrovascular dysfunction remain to be clarified. In this study, we demonstrated that dietary curcumin significantly decreased ROS production and improved cerebrovascular endothelium-dependent relaxation in aging rats. Most significantly, we demonstrated for first time that curcumin administration increased cerebrovascular AMPK phosphorylation and UCP2 levels both *in vitro* and *in vivo*. These beneficial effects of curcumin were absent in UCP2 knockout mice.

In summary, our findings provide the first evidence that chronic pharmacological AMPK/UCP2 pathway activation by curcumin treatment may be an effective therapeutic strategy to reverse age-related cerebrovascular dysfunction. Curcumin administration may represent a promising lifestyle intervention for preventing age-related cerebrovascular disturbances.

**Conflict of Interest**

No potential conflicts of interest were disclosed.

**Acknowledgements**

We thank Lijuan Wang (Chongqing Institute of Hypertension, China) for technical assistance. This research was supported by grants from the National Basic Research Program of China (2012CB517805 and 2012CB517806) and National Natural Science Foundation of China (81130006).
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