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Curcumin attenuates hyperglycaemia-mediated AMPK activation and oxidative stress in cerebrum of streptozotocin-induced diabetic rat

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Abstract

Oxidative stress has been strongly implicated in the pathogenesis of diabetic encephalopathy (DE). Numerous studies have demonstrated a close relationship between oxidative stress and AMPK activation in various disorders, including diabetes-related brain disorders. Since curcumin has powerful antioxidant properties, this study investigated its effects on hyperglycaemia-mediated oxidative stress and AMPK activation in rats with DE. Diabetes was induced by a single intraperitoneal injection of streptozotocin (STZ-55 mg/kg BW). The diabetic rats were then orally administered curcumin (100 mg/kg BW) or vehicle for 8 weeks. The cerebra of the diabetic rats displayed upregulated protein expression of AdipoR1, p-AMPKα1, Tak1, GLUT4, NADPH oxidase sub-units, caspase-12 and 3-NT and increased lipid peroxidation in comparison with the controls and all of these effects were significantly attenuated with curcumin treatment, except for the increase in AdipoR1 expressions. These results provide a new insight into the beneficial effects of curcumin on hyperglycaemia-mediated DE, which are produced through the down-regulation of AMPK-mediated gluconeogenesis associated with its anti-oxidant property.

Keywords: *Curcumin, diabetic encephalopathy, oxidative stress, apoptosis, AMPK*

Introduction

Diabetes, a chronic metabolic disorder, is associated with micro-vascular complications, including retinopathy, nephropathy and peripheral neuropathy [1]. In addition to the peripheral nervous system complications that are commonly reported in diabetic patients, many studies have demonstrated that diabetes can also have a negative impact on the central nervous system [2–8]. Type 1 diabetes mellitus is associated with the gradual development of end-organ damage in the central nervous system [9]. This little-known complication, which is referred to as 'diabetic encephalopathy (DE)' and is characterized by impaired cognitive function and neurochemical and structural abnormalities, involves direct neuronal damage caused by high amounts of intracellular glucose as well as micro- and macro-vascular cerebral diseases, which occur during diabetic complications. However, the degree of brain damage caused by high levels of intracellular glucose remains controversial. Recent reports strongly suggest that oxidative stress plays a central role in the development of complications in diabetes-associated neuronal disorders [10,11].

When 5'-adenosine mono phosphate (AMP)-activated protein kinase (AMPK, an intracellular sensor of energy status) is activated, cellular energy content is reserved and AMPK serves as a key regulator of cell survival or death in response to pathological stress (e.g. oxidative stress, endoplasmic reticulum stress, hypoxia and osmotic stress) [12–14]. Various studies

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have reported that the activation or deactivation of AMPK has beneficial effects on diabetes-related disease conditions [15,16]. The exact mechanism by which AMPK activation controls hyperglycaemia remains controversial.

The induction of oxidative damage by hyperglycaemia eventually leads to the over-production of reactive oxygen species (ROS), which is not corrected by intracellular antioxidant systems due to the excess amount of ROS. Previous reports have suggested that the normalization of ROS production with an antioxidant agent would have a beneficial effect on hyperglycaemia-related disorders [17]. Antioxidants have been shown to exert significant protective effects against various neurodegenerative disorders associated with diabetes and oxidative stress [18,19].

Curcumin contains flavonoids in its structure, is derived from *Curcuma longa* and has many pharmacological actions, including antioxidant [20], anti-inflammatory [21] and anti-cancer properties [22]. Oral supplementation with curcumin has been shown to have neuroprotective effects against heavy metal-induced toxicity [23] as well as diabetic neuropathy [24]. In addition, it has also been reported that curcumin has stronger scavenging activity against superoxide and hydroxyl radicals than the natural antioxidant vitamin E [25]. Recently, Kuhad and Chopra [26] provided behavioural and biochemical evidence for curcumin being able to attenuate DE induced by the intraperitoneal (i.p.) injection of STZ in rats.

Although curcumin has been demonstrated to have powerful antioxidant effects on various disorders, the effects of curcumin on the activation of AMPK during chronic hyperglycaemia-related DE disorders is still unclear. Therefore, we investigated the effects of curcumin on hyperglycaemia-induced oxidative stress in rats with DE.

Materials and methods

Materials

Unless otherwise stated, all reagents were of analytical grade and purchased from Sigma (Tokyo, Japan). Curcumin was purchased from Sigma Aldrich (St Louis, MO).

Diabetes induction

Diabetes was induced by a single i.p. injection of STZ (Sigma, St Louis, MO) at a dose of 55 mg/kg body weight (BW) to 8–10-week-old male Sprague Dawley rats, which were obtained from Charles River Japan Inc. (Kanagawa, Japan). The STZ was dissolved in 20 mM sodium citrate saline buffer (pH 4.5) and injected within 5 min of preparation. Age-matched male Sprague Dawley rats were injected with 100 μl of citrate buffer and were used as non-diabetic normal rats. The rats were allowed free access to water and chow throughout the study period and were treated in accordance with the guidelines for animal experimentation of our institute.

Experimental protocol

At 3 days after the STZ injection, the rats' blood glucose (BG) levels were measured using Medi-safe chips (Terumo Inc., Tokyo, Japan). The rats with blood glucose levels > 300 mg/dL were considered to be diabetic and included in this study. The rats were divided into the following three groups: (1) the vehicle-treated normal (non-STZ induced) group (NG; $n = 6$), (2) the vehicle-treated diabetic group (DG; $n = 6$) and (3) the curcumin-treated diabetic group (CG; $n = 10$). Curcumin was suspended in 0.5% gum arabic and administered orally for 8 weeks at a dose of 100 mg/kg BW. The NG and DG rats received 0.5% HEC alone. At 8 weeks after the STZ injection or curcumin treatment, the rats were anaesthetized with a single i.p. injection of pentobarbital (50 mg/kg BW) and then their brains were excised and the cerebrum was separated from the brain. The cerebrum was immediately snap-frozen in liquid nitrogen and stored at -80°C until the protein and biochemical analyses.

Western blotting

Protein lysate was prepared from the cerebrum as described previously [27]. The total protein concentration of the samples was measured by the bicinchoninic acid (BCA) method. For the determination of the protein levels of AdipoR1; p-AMPKα1; Tak1; NADPH oxidase sub-units, such as p67phox and gp91phox; GLUT4; caspase12; and 3-NT, equal amounts of protein extract (50 μg) were separated by sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis (Bio-Rad, CA, USA) and electrophoretically transferred to nitrocellulose membranes. The membranes were then blocked with 5% non-fat dried milk in Tris buffered saline Tween (20 mM Tris (pH 7.6), 137 mM NaCl and 0.1% Tween 20). Primary antibodies against AdipoR1 (sc-46749), p-AMPK α1 (sc-101630), Tak1 (sc-166562), p67phox (sc-7663), gp91phox (sc-5827), 3-NT (sc-65385), GLUT4 (sc-53566) and caspase12 (sc-5627) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Primary antibody against β-actin (#4970) was obtained from Cell Signaling Technology Inc. (Beverly, MA).

All the antibodies were used at a dilution of 1:1000. The membrane was incubated overnight at 4°C with the primary antibody and the bound antibody was visualized using the respective horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology Inc.) and chemiluminescence developing agents (Amersham Biosciences, Buckinghamshire,

Table I. Curcumin in lipid peroxidation of STZ-induced diabetic cerebrum.

Group	MDA level (nmol/mg protein)
NG	0.151 ± 0.017
DG	0.500 ± 0.023**
CG	0.218 ± 0.018#

Results are presented as mean ± SEM. MDA = malondialdehyde; NG, age-matched vehicle-treated rat; DG, vehicle-treated STZ-induced diabetic rat; CG, curcumin (100 mg/kg/day) treated STZ-induced diabetic rat. $^{**}p < 0.01$, when compared DG vs NG; $^{\#}p < 0.05$, when compared DG vs CG.

UK). The level of β-actin was estimated in each sample to ensure equal sample loading. The films were scanned and band densities were quantified by densitometric analysis using the Scion Image program (Epson GT-X700, Tokyo, Japan). All values were normalized by setting the density of the normal samples to 1.0.

Lipid peroxidation (LPO)

LPO was assessed by measuring the thiobarbituric acid (TBA) reactivity of malondialdehyde (MDA), an end product of fatty acid peroxidation. For this purpose, the cerebrum was rinsed, weighed, resuspended at 50 mg/ml in 0.01 M PBS (pH 7.2), homogenized and analysed using the thiobarbituric acid-reacting substances (TBARS) assay kit (Oxitek, ZeptoMetrix Corporation, New York, USA) [27].

Results

Reduction in LPO by curcumin treatment

The formation of malondialdehyde (MDA) is an early indicator of the induction of oxidative stress. In this study, we found that the cerebra of diabetic rats showed increased MDA levels in comparison to vehicle treated rats and that these increases were significantly reduced by curcumin treatment (Table I).

Curcumin attenuates the expression of NADPH oxidase sub-units, 3-NT and caspase12

Chronic exposure to hyperglycaemia leads to oxidative stress and nitrosamine stress, which eventually lead to cerebral apoptosis. In this study, cerebra from diabetic rats showed significantly increased oxidative stress and nitrosamine stress, which were confirmed by the up-regulated protein expression of p67phox, gp91phox and 3-NT. In addition, the diabetic cerebra showed increased caspase12 protein expression. Therefore, it appears that endoplasmic reticulum stress is involved in the hyperglycaemia-mediated changes seen in the cerebra of these rats. Treatment with curcumin significantly normalized the expression levels of p67phox, gp91phox, 3-NT and caspase-12 in the cerebra of the STZ-induced diabetic rats (Figures 1 and 2).

Regulation of Tak1, p-AMPKα1, GLUT4 and AdipoR1 by curcumin treatment

Oxidative stress displays a close relationship with AMPK activation and the diabetic rat cerebra showed up-regulated protein expression levels of Tak1 and p-AMPKα1. Curcumin, a powerful antioxidant, significantly down-regulated Tak1 and p-AMPKα1 expression. The diabetic rats showed increased expression of the glucose transporter GLUT4 compared with the normal and curcumin treatment normalized GLUT4 protein expression. Finally, diabetes significantly increased the protein expression of AdipoR1 in the cerebrum, but curcumin treatment did not adequately attenuate cerebral AdipoR1 protein expression (Figures 2 and 3).

Discussion

A large number of studies have reported that diabetes patients are more vulnerable to cognitive impairment than normal people. Brands et al. [9] reported that type 1 diabetes is strongly associated with end-organ damage in the central nervous system, which is characterized by electrophysiological and neuroradiological changes and is referred to as diabetic encephalopathy (DE). Furthermore, Biessels et al. [3] provided evidence that diabetic patients face a greater risk of Alzheimer's disease. One of the potential mechanisms of hyperglycaemia-mediated brain damage is the induction of oxidative stress in diabetes patients. Studies have shown that the i.p. injection of STZ in rats causes impaired cognitive function through the activation of oxidative stress and nitrosative stress [26]. So, we examined the role of curcumin, a potent antioxidant, in hyperglycaemia-mediated oxidative stress and the activation of AMPK in the rat cerebrum.

Excessive oxidative stress is widely acknowledged to play an important role in the development of diabetes-related disorders. Diabetes is usually accompanied by the increased production of free radicals, including reactive oxygen and nitrogen species or an impaired antioxidant defense mechanism. Glucose oxidation is believed to be the major source of reactive oxygen species (ROS) in diabetes patients. In addition, hyperglycaemia could be the major culprit in mitochondrial dysfunction, which leads to increased production of superoxide anions [1]. The non-protein thiol (NP-SH) levels in mitochondria play an important role in controlling ROS generation. Chronic hyperglycaemia alters the level of NP-SH in mitochondria, making them more vulnerable to oxidant species. Kamboj and Sandhir [28] postulated that a decrease in the NP-SH

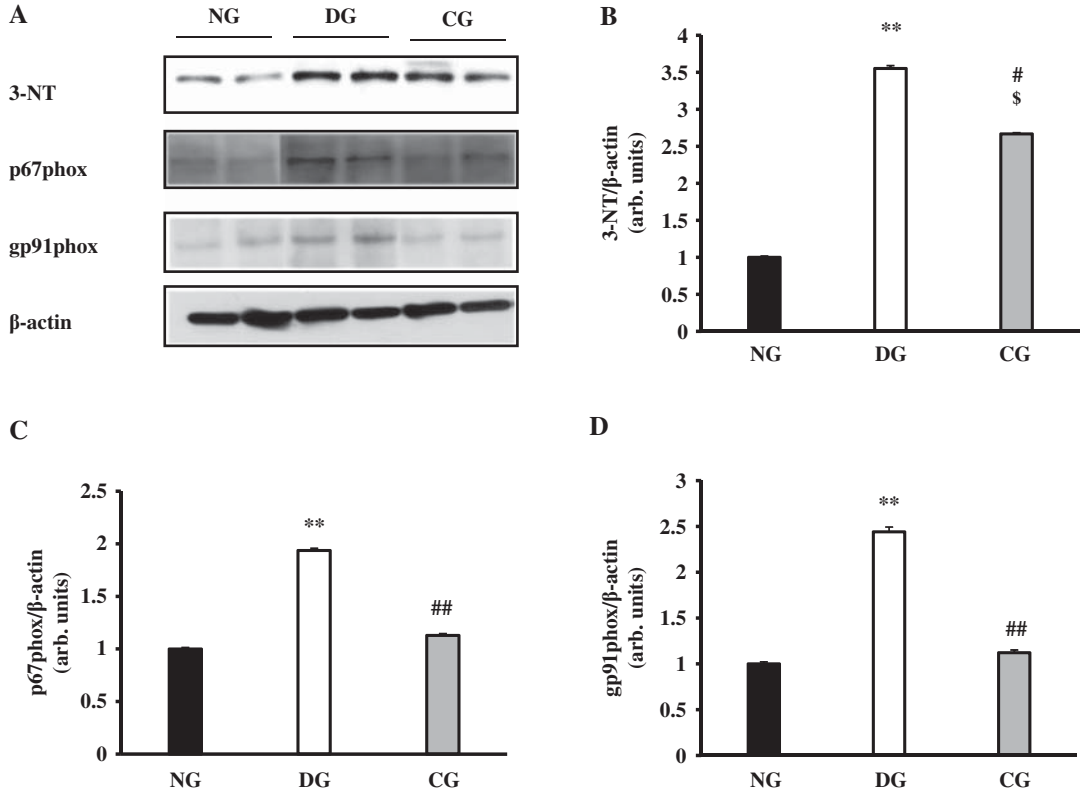

Figure 1. Effect of curcumin on the cerebral expression of 3-NT, p67phox and gp91phox in the DG cerebrum. Representative Western blots (A) showing specific bands for 3-NT, p67phox, gp91phox and β-actin as an internal control and quantified by using densitometric analysis (B–D). An equal amount of protein sample obtained from cerebrum homogenate was applied to each lane. In the DG cerebrum, curcumin treatment significantly reduced the protein expression of 3-NT, p67phox and gp91phox. NG, vehicle-only treated group; DG, STZ-induced diabetic group; CG, curcumin (100 mg/kg, BW) treated diabetic group. All values are expressed as the mean ± SEM; $n = 5$; $^{*}p < 0.01$ and $^{**}p < 0.001$ vs NG, $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ and $^{\#\#\#}p < 0.001$ vs DG, $^{\$}p < 0.05$, $^{\$\$}p < 0.01$ and $^{\$\$\$}p < 0.001$ vs NG.

level contributes to the enhanced generation of ROS in the cerebral cortices of STZ-induced diabetic rats. The production of ROS is tightly regulated in healthy cells, but their over-production during metabolic dysfunction leads to cellular injury and, eventually, to apoptosis [29]. Both *in vivo* and *in vitro* studies have reported that the increased production of ROS in the brain due to a high glucose concentration eventually leads to lipid oxidation and neuronal cell death [30,31]. These free radicals exist in a highly active state and can attack proteins, lipids and DNA.

LPO in STZ-induced diabetic rats provides indirect evidence of intensified free radical production. Also, a number of antioxidants, including nicotinamide, melatonin and vitamins C and E, have been demonstrated to normalize free radical production in diabetes-related disorders. [32]. Pierrefiche et al. [33] reported that melatonin, an antioxidant, normalized TBARS-induced LPO in the mouse brain. In addition, LPO induction causes the loss of critical components in mitochondria, which ultimately leads to mitochondria-dependent apoptosis [34]. Consistent with previous reports, the cerebra from the STZ-induced diabetic rats showed significantly increased LPO compared with the vehicle-treated group and this increase was normalized by curcumin treatment. Furthermore, numerous reports have stated that NADPH oxidase is a major source of superoxide radical production under various stress conditions. NADPH oxidase has several catalytic sub-units, such as gp91phox, and regulatory sub-units, such as p22phox, p47phox and p67phox, and Rac1 [35]. The expression of NADPH oxidase sub-units might be increased by oxidative stress in STZ-induced diabetic animal models. The blood–brain barrier (BBB) is sensitive to oxidative stress caused by the formation of a high level of free radicals; therefore, it might be damaged by the excess production of NADPH oxidase. Arias et al. [36] reported that mitochondrial impairment induced by beta-amyloid leads to the generation of ROS through the activation of NADPH oxidase sub-units [36]. It appears that chronic hyperglycaemia-induced oxidative stress generates mitochondrial ROS through the activation of NADPH oxidase sub-units, which in turn leads to the loss of mitochondrial function [27,36]. Recently, Al-Shabrawey et al. [35] reported that inhibiting NADPH oxidase or deleting NOX2 or gp91phox blocked diabetes-induced leukocyte adhesion to the vessel wall and prevented the breakdown of the blood–retinal barrier. In addition, inhibiting NADPH oxidase activity in diabetic animals could be an efficient means of reducing diabetes-related complications. In this study, curcumin treatment greatly attenuated the expression of the NADPH oxidase catalytic sub-unit

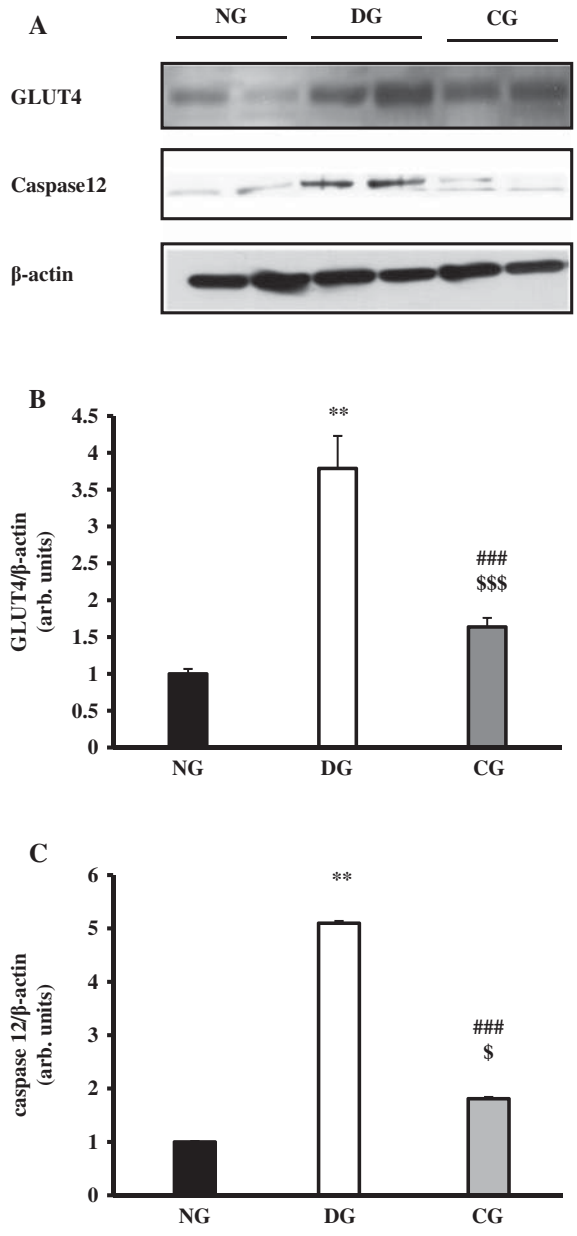

Figure 2. Effect of curcumin on the cerebral expression of GLUT4 and caspase12 in the DG cerebrum. Representative Western blots (A) showing specific bands for GLUT4, caspase12 and β-actin as an internal control and quantified by using densitometric analysis (B and C). An equal amount of protein sample obtained from cerebrum homogenate was applied to each lane. In the DG cerebrum, curcumin treatment significantly reduced the protein expression of GLUT4 and caspase12. NG, vehicle-only treated group; DG, STZ-induced diabetic group; CG, curcumin (100 mg/kg, BW) treated diabetic group. All values are expressed as the mean ± SEM; $n = 5$; $^{*}p < 0.01$ and $^{**}p < 0.001$ vs NG, $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ and $^{\#\#\#}p < 0.001$ vs DG, $^{\$}p < 0.05$, $^{\$\$}p < 0.01$ and $^{\$\$\$}p < 0.001$ vs NG.

gp91phox and the regulatory sub-unit p67phox in the STZ-induced diabetic rat brain. From the above results, it is clear that oxidative stress is induced in the STZ-induced diabetic rat brain and it is suggested that curcumin could have beneficial effects on diabetes-related brain disorders.

The induced oxidative stress is expected to be closely linked with AMPK, which is a serine-threonine kinase that acts as a key regulator of cellular energy metabolism. It is a heterotrimeric enzyme that consists of an α catalytic sub-unit and β and γ regulatory sub-units [37]. AMPK is activated by the phosphorylation of its α sub-unit by various kinases, including LKB1 [38], CaMKKβ [39] and transforming-growth factor-β–activated kinase (Tak1) [40]. AMPK activation is mainly regulated by the cellular AMP/ATP ratio [41], Ca^{2+} concentration [39] and ROS [42]. During oxidative stress, there could be an imbalance in the AMP/ATP ratio, which in turn would result in the phosphorylation of the AMPK α sub-unit. Furthermore, the generation of mitochondrial ROS during oxidative stress might be associated with AMPK activation. It has been reported that the generation of mitochondrial ROS causes AMPK activation in hypoxic conditions [43]. Thus, it appears that the generation of mitochondrial ROS during hyperglycaemia-induced oxidative stress might stimulate AMPK in the cerebrum. In this study, the cerebra of the diabetic rats showed increased AMPKα1 phosphorylation compared with the vehicle-treated group and this effect was significantly attenuated by oral supplementation with curcumin. In addition, the curcumin treatment attenuated the up-regulated expression of Tak1, an AMPK upstream kinase, in the diabetic rat cerebrum. AMPK was initially found to be an important regulator of fatty acid oxidation, but it has recently emerged that it is also an important mediator of glucose metabolism. The mechanisms through which AMPK modulates glucose uptake are only partially understood. GLUT4, the most abundant glucose transporter, resides in intracellular vesicles under normal conditions and is translocated to the plasma membrane in response to insulin, ischemia and hypoxia conditions [44,45]. AMPK activation caused increased glucose translocation by increasing the expression of the glucose transporter GLUT4 in the diabetic cerebrum compared to that in the vehicle-treated cerebrum and this effect was significantly attenuated by curcumin treatment. Estimations of the blood glucose concentration also revealed that the curcumin treatment greatly attenuated the glucose level (data not shown) in the diabetic rats. Recently, Kim et al. [46] reported that curcumin treatment significantly attenuated gluconeogenic gene expression in hepatoma cells. Our results are consistent with previous results suggesting that curcumin plays a positive role in regulating glucose metabolism by decreasing GLUT4 protein expression in hyperglycaemia-mediated brain disorders, which might be brought about through the decreased phosphorylation of AMPKα1.

There is a controversy regarding the effects of the activation or deactivation of AMPK under various conditions. Culmsee et al. [15] reported that the AMPK expression during brain development in rats was high and induces neuronal survival under glucose

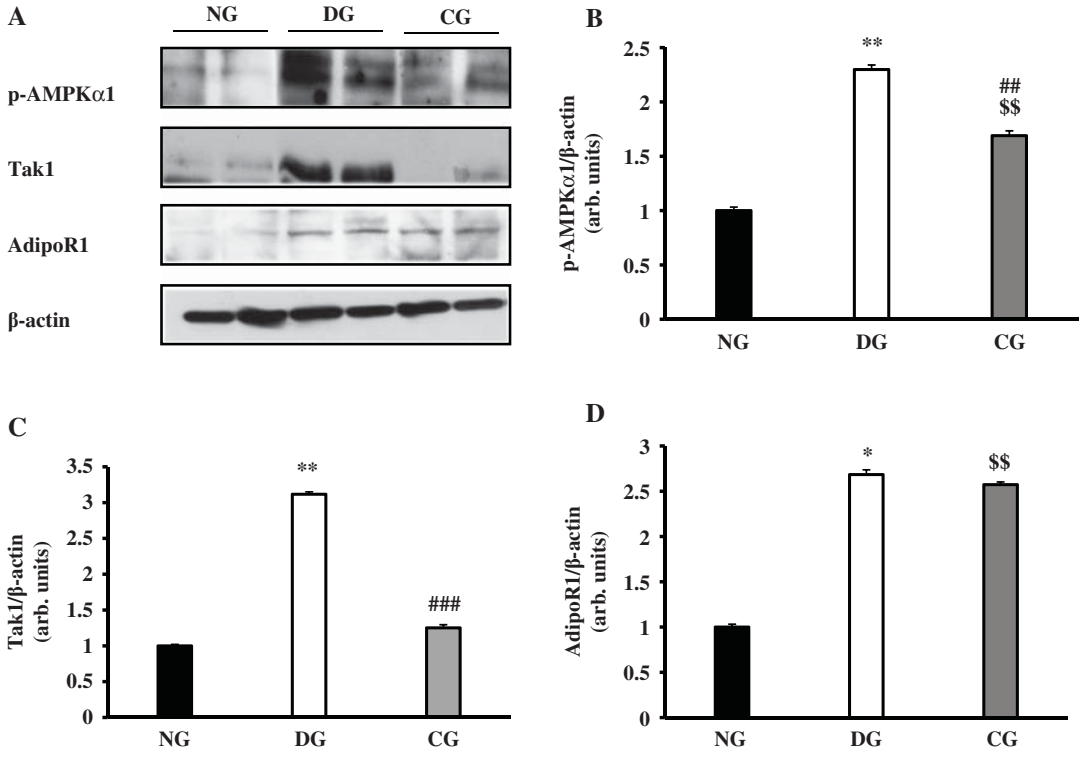

Figure 3. Effect of curcumin on the cerebral expressions of p-AMPKα1, Tak1 and AdipoR1 protein in the DG cerebrum. Representative Western blots (A) showing specific bands for p-AMPKα1, Tak1, AdipoR1 and β-actin as an internal control and quantified by using densitometric analysis (B–D). An equal amount of protein sample obtained from cerebrum homogenate was applied to each lane. In the DG cerebrum, curcumin treatment significantly reduced the phosphorylation of AMPKα1 and Tak1 protein expression. NG, vehicle-only treated group; DG, STZ-induced diabetic group; CG, curcumin (100 mg/kg, BW) treated diabetic group. All values are expressed as the mean ± S.E.M; $n = 5$; $^{*}p < 0.01$ and $^{**}p < 0.001$ vs NG, $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ and $^{\#\#\#}p < 0.001$ vs DG, $^{\$}p < 0.05$, $^{\$\$}p < 0.01$ and $^{\$\$\$}p < 0.001$ vs NG.

deprivation, but McCullough et al. [16] reported that following ischemia AMPK activation promotes damage to hippocampal and cortical neurons. Dagon et al. [47] reported that AMPK activation by diet restriction (DR) had different outcomes on neural cells. Sixty per cent DR had an anti-apoptotic effect, reversed the AMP/ATP ratio and induced AMPK activation, whereas DR to 40% promoted neural cell apoptosis via severe energy depletion associated with an irreversible AMP/ATP ratio [47]. Furthermore, oxidative damage induces membrane permeability, which in turn increases the expression of cytochrome *c* in the cytosol, which induces further apoptosis [48]. It has been reported that STZ-induced diabetic rat brains show increased expression of cytochrome *c* and apoptosis markers, such as caspase3 [28]. In this study, we observed the increased expression of caspase12, an apoptosis marker, in the diabetic cerebrum compared with that in the vehicle-treated cerebrum and curcumin treatment significantly attenuated the expression of caspase12 in the diabetic cerebrum. These results suggest that hyperglycaemia-induced diabetes causes severe oxidative stress associated with AMPK activation and that the AMP/ATP ratio can not be reversed in these conditions. This could lead to cerebral apoptosis. Curcumin treatment significantly reduces oxidative stress by reversing of the AMP/ATP ratio via the down-regulation of AMPK phosphorylation; therefore, it protects the cerebrum against apoptosis.

Our results strongly indicate that diabetes induced by STZ injection cause increased oxidative stress in the cerebrum through the excessive production of free radicals associated with the up-regulation of NADPH oxidase sub-unit expression. This increased oxidative stress is considered to stimulate the phosphorylation of AMPK, which in turn promotes cerebral apoptosis. Curcumin, a potent antioxidant, could be beneficial for ameliorating diabetes-induced brain disorders, mainly by attenuating oxidative stress as well as AMPK phosphorylation in the cerebrum.

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Declaration of interest

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