Quercetin protects rat dorsal root ganglion neurons against high glucose-induced injury in vitro through Nrf-2/HO-1 activation and NF-κB inhibition

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Aim: To examine the effects of quercetin, a natural antioxidant, on high glucose (HG)-induced apoptosis of cultured dorsal root ganglion (DRG) neurons of rats.

Methods: DRG neurons exposed to HG (45 mmol/L) for 24 h were employed as an in vitro model of diabetic neuropathy. Cell viability, reactive oxygen species (ROS) level and apoptosis were determined. The expression of NF-κB, IκBα, phosphorylated IκBα and Nrf2 was examined using RT PCR and Western blot assay. The expression of hemeoxygenase-1 (HO-1), IL-6, TNF-α, iNOS, COX-2, and caspase-3 were also examined.

Results: HG treatment markedly increased DRG neuron apoptosis via increasing intracellular ROS level and activating the NF-κB signaling pathway. Co-treatment with quercetin (2.5, 5, and 10 mmol/L) dose-dependently decreased HG-induced caspase-3 activation and apoptosis. Quercetin could directly scavenge ROS and significantly increased the expression of Nrf-2 and HO-1 in DRG neurons. Quercetin also dose-dependently inhibited the NF-κB signaling pathway and suppressed the expression of iNOS, COX-2, and proinflammatory cytokines IL-6 and TNF-α.

Conclusion: Quercetin protects rat DRG neurons against HG-induced injury in vitro through Nrf-2/HO-1 activation and NF-κB inhibition, thus may be beneficial for the treatment of diabetic neuropathy.

Keywords: quercetin; antioxidant; diabetic neuropathy; dorsal root ganglion; reactive oxygen species; Nrf-2; hemeoxygenase-1; NF-κB; proinflammatory cytokine
detoxifying enzymes, such as glutathione S-transferase, UDP glucuronosyltransferase, heme oxygenase-1 (HO-1), NADPH
quinone oxidoreductase, and microsomal epoxide hydrolase. A build-up of phase I metabolites can wreak havoc on cells. The transcription factor nuclear factor (erythroid-derived 2)-like 2 (Nrf-2) is recognized as a critical factor in the induction of phase II enzymes.[8]

Oxidative stress results in the activation of NF-kB, which triggers the activation of inflammatory signaling pathways[7]. NF-kB is a rapid-acting pleiotropic transcription factor that regulates a diverse group of genes, including genes that encode inducible nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2), and proinflammatory cytokines. In experimental diabetic neuropathy models, ROS-activated NF-kB and its oxidant products have been shown to enhance the expressions of iNOS, COX-2, tumor necrosis factor-alpha (TNF-α), and interleukin (IL)-6[9–11]. Oxidative stress and inflammation have been shown to interact with each other and to be inseparably linked to diabetic neuropathy.

Treatments that are currently prescribed to lessen neuropathic pain have undesirable secondary effects and situational variable efficacy[12]. Therefore, researchers are searching for better diabetic neuropathy drug options. Flavonoids are an attractive treatment option, given their efficacy in clearing ROS and their ability to protect against neuronal damage[13]. The flavonoid quercetin is the most potent scavenger of ROS, and suppresses iNOS activity, consequently reducing damage from nitric oxide superoxide anions[14]. Furthermore, quercetin reduces low-density lipoprotein oxidation and suppresses iNOS activity, consequently reducing damage from nitric oxide superoxide anions[15].

The axons of dorsal root ganglion neurons (DRG neurons) are afferents that relay sensory information to the central nervous system. With their exuberant metabolism and sensitivity to high glycemia, DRG neurons are an experimental target of diabetic neuropathies[16, 17]. Even short-term hyperglycemia can produce oxidative damage and apoptosis in DRG neurons[17]. Inflammatory factors play an important role in DRG neuronal damage, especially in painful neuropathy[9, 18, 19]. In previous studies, lower glucose concentrations induced DRG neuron death in culture, whereas total glucose levels above 35 mmol/L produced hyperglycemic insult leading to DRG neuron death[20]. To produce a hyperglycemic insult, 20 mmol/L additional glucose (yielding a total 45 mmol/L glucose) were added to the media for the period specified in individual experiments.

In this study, we evaluated the effects of quercetin (2.5, 5, and 10 mmol/L) on high glucose (HG)-treated DRG neurons, in terms of cell viability, ROS levels, and apoptosis. The expression levels of NF-kB, Nrf2/HO-1, proinflammatory mediators (IL-6 and TNF-α), iNOS, COX-2, and caspase-3 were also evaluated.

Materials and methods

Cell culture and treatments

Unless otherwise noted, cell culture materials and other reagents were obtained from Sigma (St Louis, MO, USA). DRG neurons were harvested from Sprague-Dawley rats (Vital River Company, China) at embryonic d 15. Prior to their establishment in culture, the neurons were digested with 0.25% trypsin in D-Hanks’ solution at 37°C for 20 min. After digestion and dissociation, fetal bovine serum (Invitrogen, Grand Island, NY, USA) was added to a final concentration of 10% to stop digestion. Cells were passed through a 74-μm filter and centrifuged for 5 min at 201.24×g. The pellet was resuspended in neurobasal medium (Invitrogen, Grand Island, NY, USA) of 2% B-27 supplement without antioxidants (Invitrogen, Grand Island, NY, USA), 10 ng/mL nerve growth factor (Merck-Millipore, Darmstadt, Germany), 1 mmol/L L-glutamine, and 1000 U/mL of penicillin/streptomycin/neomycin (ABX, North China Pharmaceutical Group Corp, Shijiazhuang, China) solution.

Dissociated DRG cells were cultured in poly-D-lysine precoated 96-well or 6-well clusters (Costar, Corning, NY) at 5×10^4 cells/well in 0.1 mL for 96-well clusters or 2×10^5 cells/well in 2 mL for 6-well clusters. DRG cells were cultured in media at 37°C with 5% CO₂ and maintained in media containing 20 μmol/L FUDR for another 24 h to inhibit the growth of non-neuronal cells. The purity of neuronal cells was confirmed by fluorescent labeling of microtubule-associated protein 2 (MAP-2, Merck-Millipore) and a 4′,6-diamidino-2-phenylindole (DAPI, Zhongshan Golden Bridge Biotechnology Co, Ltd, Beijing, China) fluorescent counterstain (Figure 1). Neurons were cultured for an additional 24 h under different experimental conditions before examination.

Figure 1. Double fluorescent labeling of MAP-2 and DAPI. MAP-2-labeled DRG neurons, DAPI-labeled nuclei of all cells in culture. (Scale bar=75 μm).

Neurobasal medium was used as the control group medium because its glucose concentration is 25 mmol/L, which is optimal for the survival and growth of DRG neurons. An additional 20 mmol/L glucose was added to the medium for the HG groups[17]. The cells were randomly divided into five groups: control (CON, 25 mmol/L glucose), HG (45 mmol/L glucose), and HG plus 2.5 mmol/L quercetin (Q2.5), 5 mmol/L quercetin (Q5), or 10 mmol/L quercetin (Q10). The choice of quercetin concentrations was based on our unpublished results.
Cell viability assay

DRG neurons were plated into 96-well clusters at a density of 5×10^4 cells/well. After a 24-h incubation under group-specified experimental conditions, the clustered neurons were processed for detection of cell viability by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays. Briefly, 100 μL of 5 mg/mL stock solution of MTT was added to each well and incubated at 37 °C for 4 h, after which 100 μL of dimethyl sulfoxide (DMSO) was added to each well. Absorbance was measured at 570 nm with a microplate reader. Cell viability was expressed as the ratio of clustered neurons to control cells.

Determination of apoptotic cells

After a 24-h incubation (2×10^6 cells/well) on poly-D-lysine–coated coverslips in 6-well plates according to group specifications, apoptotic cells were detected by the terminal deoxynucleotidyltransferase-mediated UTP nick end-labeling (TUNEL) method with an in situ cell death detection kit (Roche, Mannheim, Germany), according to the manufacturers’ instructions. After detection by fluorescence microscopy at an excitation wave length of 530 nm, the cells were added to a converter-POD, stained with diaminobenzidine (DAB, Zhongshan Golden Bridge Biotechnology Co, Ltd, Beijing, China), counterstained with hematoxylin, and examined by light microscopy. Cells treated with DNase I (Fermentas, Burlington, Canada) were used as positive controls. Cells labeled with the reaction mixture, but without terminaltransferase, were used as negative controls. Apoptotic cells were examined under a light microscope; at least 500 cells from each group were examined in a blinded manner.

Measurement of ROS

Intracellular ROS was measured with the cell-permeable oxidation-sensitive fluorescent probe 2′,7′-dichlorofluorescein diacetate (DCFH-DA, Molecular Probes, and Eugene, OR). DCFH-DA freely diffuses into cells, where it is cleaved into DCFH by intracellular esterase, which cannot pass out of the cell. In the presence of ROS, DCFH becomes oxidized into a fluorescent product, DCF, which serves as an index to indicate intracellular ROS levels. DCFH-DA was dissolved freshly in neurobasal medium and used at a final concentration of 5 μmol/L. Intracellular ROS levels were elevated from 1 h, peaked at 4 h, and thereafter declined; therefore, 4 h was chosen as the time point for analysis. After 4 h of HG treatment, the culture medium was removed, and the cells were washed with 0.1 mmol/L phosphate-buffered saline (PBS, pH 7.4, Invitrogen) three times to remove excess probe. Fluorescence intensity was analyzed by flow cytometry with histogram plots. ROS values were calculated relative to unlabeled control cells.

Quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR)

Total RNA was extracted with the TRIzol® RNA isolation reagent (Invitrogen). Reverse transcription was performed with a PrimeScript® RT Master Mix kit (TAKARA Biotechnology Co, Ltd, Dalian). The cDNA products were subjected to real-time PCR with the SYBR premixed system and specific primers. Real-time PCR was performed in a StepOnePlus™ Real-Time PCR System (Applied Biosystems) by using the following program: 95°C for 10 min, followed by 40 cycles of 95°C for 15 s, and 60°C for 1 min. Each sample was analyzed in triplicate. β-actin mRNA was quantified in each sample as an endogenous control. The sequences of the primers used are summarized in Table 1. Nucleotide BLAST and melting curve analyses were used to confirm primers specificity.

Table 1. Primer and probe sequences.

| Protein     | Reference | Direction | Sequence                  |
|-------------|-----------|-----------|---------------------------|
| Nrf-2       | 21        | Sense     | 5′-GCAACTCCAGAAGGAACAGGG-3′ |
|             |           | Antisense | 5′-GGATTGTCTGCCAAAAGGC-3′  |
| HO-1        | 22        | Sense     | 5′-CTCTCAGAGGGTGAGTGC-3′   |
|             |           | Antisense | 5′-TGGTGTGGTTGCTCTTAC-3′   |
| NF-kB       | 23        | Sense     | 5′-CATGGGTTCGTTCAAGTGCGA-3′|
|             |           | Antisense | 5′-TGCGTGGCTTGTTAGTGC-3′   |
| IL-6        | 24        | Sense     | 5′-CAGGGAGACTCGGAAGAAAGTA-3′|
|             |           | Antisense | 5′-AATCCCGAGAGCCAGAAGCA-3′ |
| TNF-α       | 25        | Sense     | 5′-TACTGAACTGCGGGGTGATTG-3′|
|             |           | Antisense | 5′-CAGCTCTGTGCTTTGGAGAAGA-3′|
| iNOS        | 26        | Sense     | 5′-TGAACCTTACGGACACGACCA-3′|
|             |           | Antisense | 5′-TCAATGTAAGTGGATCTTGCT-3′|
| COX-2       | 27        | Sense     | 5′-TGATGGAGACTACGTCGAACAC-3′|
|             |           | Antisense | 5′-CCGCTACCTCGTGCTGTAATGACC-3′|
| Caspase-3   | 28        | Sense     | 5′-GCCGAAACTCTCATTGATCG-3′  |
|             |           | Antisense | 5′-CATATCATCGTCATGCCACTGTC-3′|

Western blot analysis

Total protein was isolated with protein lysis buffer supplemented with phenylmethylsulfonyl fluoride (Beyotime Institution of Biotechnology, Haimen, China) and protease inhibitor (AmrescoLLC, Solon, OH, USA). Protein samples were separated by 10% SDS-PAGE (Beyotime Institution of Biotechnology) and transferred to NC membranes (Pall Gelman Laboratory, Ann Arbor, MI, USA). Subsequently, the membranes were blocked with 5% fat-free milk and incubated with primary antibodies against iNOS, COX-2, NF-kB (p65), IκBα, phospho IκBα, and β-actin (Santa Cruz Biotechnology, Santa Cruz, CA, USA), Nrf-2, HO-1, and caspase-3. All Santa Cruz antibodies were used at a dilution of 1:200. Anti-Nrf-2 was used at 1:500, and anti-HO-1 and anti-caspase-3 were used at 1:1000.

For immunodetection, blots were incubated with primary antibodies overnight at 4°C and then washed and incubated with horseradish peroxidase (HRP)-conjugated anti-mouse
IgG (H+L) (1:5000, Zhongshan Golden Bridge Biotechnology Co, Ltd, Beijing, China) or HRP-conjugated anti-rabbit IgG (H+L) (1:3000, Zhongshan Golden Bridge Biotechnology Co, Ltd, Beijing, China) for 1 h. ECL Super Signal® West Pico Chemiluminescent Substrate (Pierce) was applied to enable visualization of the bands. The relative levels of each protein to β-actin were determined by densitometry analysis with ImageJ software.

**Statistical analysis**

All data were expressed as the mean±standard error of the mean (SEM). Groups were compared by one-way analysis of variance (ANOVA), followed by the Student-Newman-Keuls post hoc test to detect differences among various groups or two independent-sample t-tests to compare differences between two groups. SPSS software (version 18.0) was used for all analyses. P-values <0.05 were considered statistically significant.

**Results**

**Quercetin had a protective effect on DRG neuron**

MTT reduction assays showed that the cell viability in DRG neurons exposed to HG was significantly reduced compared to that observed in control DRG neurons (Figure 2). Quercetin attenuated HG-induced decreases in cell viability in a dose-dependent manner.

**Quercetin inhibited production of HG-induced ROS**

The measurement of intracellular hydrogen peroxide levels with the DCFH-DA assay revealed that HG produced a marked increase in intracellular ROS levels, compared to that seen in control cells (Figure 3A, 3B). Quercetin blocked this increase in intracellular ROS in a dose-dependent manner.

**Quercetin prevents HG-induced apoptosis of DRG neurons**

As shown in Figure 4A–4C, TUNEL analysis showed that the percentages of apoptotic cells were increased in groups exposed to HG. The addition of quercetin reduced the frequency of apoptotic DRG neurons in HG conditions significantly, and these inhibitory effects were dose dependent.

**Effects of quercetin on the Nrf-2 and HO-1 expression**

After 24 h, Nrf-2 expression was decreased in the HG group, but increased in the HG+quercetin groups, compared with the control group. HO-1 was decreased in the HG group compared with controls. Treatment with quercetin significantly attenuated HG effects on HO-1 protein expression. Indeed, the addition of quercetin normalized levels of Nrf-2 and HO-1 under HG conditions in a dose-dependent manner. Similar patterns of Nrf-2 and HO-1 mRNA transcription were detected across the different groups of cells (Figure 5A–5C).

**Effects of quercetin on NF-κB, IκBα, and p-IκBα levels**

The induction of inflammatory mediators under HG exposure was paralleled by the upregulation of expression of NF-κB and IκBα, and the phosphorylation of IκBα. Our Western blot experiment revealed that the levels of NF-κB, IκBα, and p-IκBα proteins were higher in cells in the HG condition than those in the control group, which indicated the activation of NF-κB. Compared to HG-exposed cells not treated with quercetin, the...
quercetin-treated and HG-exposed cells had decreased levels of NF-κB, IκBα, and p-IκBα, and these effects were dose-dependent (Figure 6A, 6B). Consistent with these Western blot protein data, our RT-PCR experiment also showed reduced levels of NF-κB mRNA in the quercetin-treated groups (Figure 6C).

Effects of quercetin on IL-6 and TNF-α expression levels
Western blot analysis showed that the IL-6 and TNF-α expression levels, which we employed as biological markers for inflammation, were increased in the HG group compared to the control group. Conversely, these markers were decreased in a dose-dependent manner in the quercetin-treated, HG-exposed cells compared to levels in the nontreated HG-exposed cells (Figure 7A, 7B). Our RT-PCR results indicated that the effects of HG and quercetin on IL-6 and TNF-α mRNA were generally consistent with the Western blot protein data (Figure 7C). However, although we observed significantly higher protein levels of TNF-α and IL-6 in the HG group than in the Q2.5 group, the mRNA transcript levels of these cytokines did not differ significantly between the HG and Q2.5 groups.

Effects of quercetin on iNOS and COX-2 expression
The expression levels of iNOS and COX-2 inducible enzymes that are abundant in cells at sites of inflammation were significantly increased in the HG group compared to the control group. Quercetin treatment reduced the expressions of iNOS and COX-2 in HG-exposed cells. This finding suggests that these enzymes likely mediate the inhibitory influence of quercetin on the activation of the inflammatory cascade. Similar to the above-described observations, the effects of quercetin on the iNOS and COX-2 levels were also dose-dependent (Figure 8A–8C).

Effect of quercetin on caspase-3 activation
Finally, to characterize the hyperglycemia-related apoptosis, relative levels of cleaved and intact caspase-3 were determined by Western blot analysis (Figure 9A, 9B) and real-time PCR (Figure 9C). We found that the inclusion of quercetin in the media significantly reduced the caspase-3 cleavage in a dose-dependent manner, although the levels of intact caspase-3 protein remained stable. Interestingly, caspase-3 mRNA transcription was increased in HG-exposed cells, but to a lesser extent than the change in cleaved caspase-3 protein expression. Treatment of HG-exposed cells with quercetin also produced a downregulation of caspase-3 mRNA transcription relative to the nontreated, HG-exposed cells. Hence, quercetin treatment appeared to counteract the influence of HG exposure on caspase activation in DRG neurons.

Discussion
In this study, we obtained evidence showing that when DRG neurons are exposed to HG, they exhibit decreased cell viability and increased levels of ROS and apoptosis. They also show increased activation of NF-κB and elevated levels of proinflammatory cytokines (IL-6 and TNF-α) and other inflammatory-related enzymes (iNOS and COX-2). Treatment with
Quercetin attenuated HG-induced apoptosis, and this effect was concomitant with an inhibition of NF-κB, Nrf-2/HO-1, and caspase-3 activity. These effects were associated with the downregulated expressions of IL-6, TNF-α, iNOS, and COX-2. The effects of quercetin on DRG neurons were dose-dependent and detectable at both the protein and mRNA levels.

The present study complements prior research showing decreases in the nuclear localization of NF-κB subunits in HG-exposed DRGs being employed as an in vitro analog of peripheral neuropathy. The model is clinically relevant, in that DRGs are nodes of sensory afferent somata, and diabetes-associated numbness and pain in patients can be attributed to dysfunction and apoptosis of DRG neurons [16]. Under these conditions, the changes in NF-κB may be attributable, at least in part, to impaired mitochondrial ROS scavenging due to a deficiency of manganese superoxide dismutase. Importantly, enhancement of NF-κB activity in cultured HG-exposed DRG neurons, similar to that shown here, has been shown by others to ameliorate suboptimal neurite outgrowth [29]. Additionally, Chiarugi [30] demonstrated that drug-dependent inhibition of neuronal NF-κB activity induced selective activation of caspase-9 and -3 and mitochondrial release of cytochrome c.

Given their high susceptibility to hyperglycemia, DRG neurons are an attractive target for diabetic neuropathy treatment. In the last decade, researchers have found that DRG neurons in culture died through programmed cell death when exposed to elevated levels of glucose, and confirmed that ROS are an important component of this induction of cell death [17]. Subsequent research revealed additional mechanisms that were contributing to this cell death, including respiratory chain dysfunction, mitochondrial DNA damage, and inflammation [9, 31, 32]. Advanced glycation end products (AGEs) and oxidized lipoproteins activate inflammatory signaling, which leads to oxidative and nitrosative stress, which in turn aggravates the inflammation [4, 33]. Therefore, clearance of oxidative and nitrosative stress and inhibition of inflammation have been considered good strategies for the treatment of diabetic neuropathy [34].
Prior studies have shown that quercetin has a good capacity for scavenging ROS and yielding neuroprotection. Quercetin has been shown to inhibit LPS-induced upregulation of both iNOS and COX-2, as well as expression of p38 mitogen-activated protein kinase (p38-MAPK), TNF-α, and NF-κB[15, 35, 36]. Additionally, quercetin has been reported to reduce DNA fragmentation, the Bax/Bcl-2 ratio, nuclear translocation of apoptosis-inducing factor, and PARP cleavage[37]. Moreover, Arredondo et al[38] recently found that quercetin could induce Nrf-2 nuclear translocation and increase the γ-glutamylcysteine ligase catalytic subunit gene expression, and these effects were associated with prevention of neuronal death from oxidant exposure. Thus, the present findings combined with prior findings suggest that quercetin works through multiple pathways to protect neurons from oxidative damage.

Researchers interested in controlling the production of free radicals are particularly interested in antioxidant enzymes, especially superoxide dismutases, catalases, and glutathione peroxidases. Nrf-2 is referred to as the “master regulator” of the antioxidant response because it modulates the expression of hundreds of genes, including not only antioxidant enzymes, but also numerous genes involved in diverse processes such as immune and inflammatory responses[39]. Under quiescent conditions, the repressor of Nrf-2, Kelch-like ECH-associated protein 1 (Keap1), binds Nrf-2 and thereby sequesters it in the cytoplasm. When Keap1 encounters ROS, it dissociates from Nrf-2, leaving Nrf-2 less prone to proteasomal degradation and free to translocate to the nucleus, where it interacts with the antioxidant response element to increase the expression of many antioxidant and detoxifying enzymes and proteins (eg, HO-1, NAD(P)H dehydrogenase quinone 1, glutathione S-transferase, and superoxide dismutase). Although brief (<3 h), mild oxidative stress stimulates nuclear transfer of Nrf-2 to produce a protective effect on DRG neurons. Long-term hyperglycemia inhibits the expression of Nrf-2 and depresses the expression of HO-1[40]. Our findings suggest that quercetin alleviates the negative long-term effects of HG by upregulating the expressions of Nrf-2 and HO-1.

NF-κB can be activated by many factors, including, in addition to hyperglycemia, hypoxia, ischemia, and infection. Exposure to HG can affect NF-κB expression through various pathways, with hyperglycemia-related ROS being a major factor[41, 42]. Hyperglycemia can trigger phosphatidylinositol-
3-kinase/akt signaling and induce NF-κB-related upregulation of COX-2, which in turn triggers prosapoptotic caspase-3 activity.[43] Other researchers have found that NADPH oxidase-related ROS-induced apoptosis is mediated via JNK-dependent activation of NF-κB in cardiomyocytes exposed to HG.[44] Additionally, AGE-induced ROS generation also triggers NF-κB activation and subsequent TNF-α production to induce apoptosis.[45] Treatment with an NF-κB inhibitor has been reported to prevent AGE-induced iNOS expression by interfering with p38 MAPK activity.[46]

In conclusion, the present experiments demonstrated that apoptosis of DRG neurons was increased under HG (diabetes model) conditions, and this effect was attenuated in the presence of quercetin. Our data further suggest that these protective effects of quercetin may be attributable to influences on the Nrf-2/HO-1 pathway, scavenging ROS, and inhibition of NF-κB activation. The possibility that NF-κB may play a critical role in the pathogenesis of neuronal dysfunction due to hyperglycemia suggests that the inhibition of local NF-κB activity may be a beneficial strategy for preventing or alleviating diabetic neuropathy complications. Furthermore, our findings of the protective effects of quercetin on DRG neurons in a hyperglycemic environment point to quercetin as a potential medicine for diabetic neuropathy.

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Author contribution

Yue SHI, Xiao-chun LIANG, and Hong ZHANG designed the research; Yue SHI and Hong ZHANG performed the research; Qun-li WU, Ling QU, and Qing SUN contributed new reagents and analytic tools; Yue SHI analyzed the data; and Yue SHI wrote the paper.

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