Acta Pharmacologica Sinica (2018) 39: 1582–1589
 1671-4083/18
www.nature.com/aps

Article

Prolonged DADLE exposure epigenetically promotes Bcl-2 expression and elicits neuroprotection in primary rat cortical neurons via the PI3K/Akt/NF-κB pathway

Min ZHU[1, 2, #], Ming LIU[1, 3, #], Qi-lin GUO[1], Cui-qing ZHU[1], Jing-chun GUO[1, *]

[1]Department of Translational Neuroscience, Jing'an District Centre Hospital of Shanghai, State Key Laboratory of Medical Neurobiology & Institutes of Brain Science, Fudan University, Shanghai 200032, China; [2]Shanghai Key Laboratory of Visual Impairment and Restoration, Eye & ENT Hospital, Fudan University, Shanghai 200032, China; [3]Shanghai High School, International Division, Shanghai 200231, China

Abstract

Both *in vivo* and *in vitro* studies have shown the beneficial effects of the delta-opioid receptor (DOR) on neurodegeneration in hypoxia/ischemia. We previously reported that DOR stimulation with [(D-Ala2, D-Leu5) enkephalin] (DADLE), a potent DOR agonist, for both a short (minutes) and long (days) time has notable protective effects against sodium azide (NaN_3)-induced cell injury in primary cultured rat cortical neurons. We further demonstrated that short-term DADLE stimulation increased neuronal survival through the PKC-mitochondrial ERK pathway. However, the mechanisms underlying long-term neuroprotection by DADLE remain unclear. Here, we showed that DOR stimulation with DADLE (0.1 μmol/L) for 2 d selectively activates the PI3K/Akt/NF-κB pathway in NaN_3-treated neurons; this activation increased Bcl-2 expression, attenuated Cyto *c* release and promoted neuronal survival. Further investigation revealed that sustained DADLE stimulation increased Bcl-2 expression by enhancing NF-κB binding to the Bcl-2 promoter and upregulating the histone acetylation levels of the Bcl-2 promoter. Our results demonstrate that prolonged DADLE exposure epigenetically promotes Bcl-2 expression and elicits neuroprotective effects in the NaN_3 model via the PI3K/Akt/NF-κB pathway.

Keywords: DADLE; NaN_3-induced neuronal injury; neuroprotection; epigenetic regulation; Bcl-2; PI3K/Akt/NF-κB pathway

Acta Pharmacologica Sinica (2018) 39: 1582–1589; doi: 10.1038/aps.2018.7; published online 24 May 2018

Introduction

Stroke is the leading cause of death and serious, long-term disability. To date, clinical strategies that help to stop the neurodegeneration following anoxic/ischemic insults remain limited. [(D-Ala2, D-Leu5) enkephalin] (DADLE) is a synthetic opioid peptide and a highly selective delta opioid receptor (DOR) agonist[1, 2]. Since the neuroprotective effects of DOR activation were first described[1], it has attracted a great deal of attention from researchers worldwide. Accumulating evidence has suggested that stimulating DOR with DADLE elicits potent neuroprotection against anoxic/ischemic injury[1, 3-5]. Therefore, DADLE may be a promising novel therapeutic candidate for treating neurodegeneration in stroke.

After the neuroprotective effects of DOR activation were recognized, subsequent studies focused on the underlying mechanism of DORs, and great progress has been made so far. The DOR is a G_i protein-coupled receptor that functions through the cAMP signaling pathway[6]. Although the details remain unclear, it is generally accepted that the mechanisms of DOR neuroprotection fall into two categories: DORs can trigger endogenous protective pathways[4, 7-9] and maintain ionic homeostasis[10-13]. In particular, it has been confirmed by multiple studies that the PKC/MAPK pathway plays a crucial role in the neuroprotective functions of DORs[4, 7, 8, 14]. Moreover, DOR activation might elicit neuroprotection via the PI3K/Akt signaling pathway[15, 16].

Our previous studies have investigated the neuroprotective effects of DADLE using a NaN_3-induced mitochondrial dysfunction model. Much to our surprise, we found that stimulating DOR with DADLE for both a short (minutes) and long (days) time showed notable protective effects on pri-

# These authors contributed equally to this work.
*To whom correspondence should be addressed.
E-mail jingchunguo@shmu.edu.cn
Received 2017-11-21 Accepted 2018-01-02

mary rat cortical neuronal cultures[8, 17]. Further investigation revealed that short-term DADLE stimulation increases neuronal survival through the PKC-mitochondrial ERK pathway in the NaN_3 model[8]. However, the mechanism underlying the protective effects of DADLE during prolonged exposure remains unclear. In this study, we will explore the possible downstream signaling pathways mediating the neuroprotective effects of sustained DOR stimulation with DADLE in the NaN_3-induced neuronal injury model.

Materials and methods

Primary culture of rat cortical neurons

Primary cortical neuron cultures were prepared as previously described[8, 17]. Briefly, after Sprague-Dawley rats (embryonic d17–18; Shanghai Experimental Animal Center, Chinese Academy of Science, Shanghai, China) were sacrificed on gestation d 17−18, the cortical neurons were carefully collected. Then, the cells were resuspended in neurobasal medium (Gibco-BRL, Life Technologies, Rockville, MD, USA) supplemented with B-27, glutamine (0.5 mmol/L), penicillin (100 U/mL) and streptomycin (100 μg/mL) and plated onto poly-*L*-lysine (100 μg/mL, Sigma, St Louis, MO, USA) pre-coated dishes at a density of 1×10^6 cells/mL. The cells were ready for drug administration on day 8. Animal experiment protocols complied with the guidelines of the Animal Care and Use Committee of Fudan University. All efforts were made to minimize the suffering of the animals.

Drug administration

Freshly prepared NaN_3 (80 μmol/L) was applied to primary cultured neurons to induce mitochondrial dysfunction. DADLE (0.1 μmol/L) or naltrindole (NTI, 0.1 μmol/L) was administered to selectively activate or inhibit DOR activity[8, 17]. LY296004 (20 μmol/L) is a selective PI3K inhibitor, and Akt inhibitor VIII (5 μmol/L) is a specific Akt inhibitor[18]. All drugs were purchased from Sigma (St Louis, MO, USA).

Lactate dehydrogenase (LDH) assay

The activities of LDH in the cultivated medium were measured with an LDH kit (Sigma, St Louis, MO, USA) and detected with a DU-640 spectrophotometer (Beckman Instruments, Inc., Fullerton, CA, USA) as previously described[8, 17]. The absorbance data were normalized to those of the control group.

Cell viability assay

Cell viability assays were performed by adding cell counting kit-8 reagent (CCK-8, Dojindo Laboratories, Japan) directly to the cell cultures. After incubation at 37 °C for 1 h, the absorbance was read by a spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) at a wavelength of 450 nm and was proportional to the number of living cells. Each experiment was repeated three times.

Cytosolic and nuclear protein preparation

Cytosolic and nuclear proteins were extracted using a nuclear extraction kit (Chemicon, Temecula, CA, USA) according to the manufacturer's protocol. Briefly, neurons were collected after drug exposure and incubated in cytoplasmic lysis buffer for 15 min. The cells were pelleted by centrifugation at 250×*g* for 5 min and resuspended in cytoplasmic lysis buffer. A syringe with a small gauge needle was used to break up the cytoplasmic membrane. The supernatant was collected after centrifugation at 8000×*g* for 20 min and used as the cytosolic fraction. The remaining nuclear pellet was resuspended in nuclear extraction buffer for 30 min, and a fresh syringe was used to disrupt the nuclei. Following centrifugation at 16 000×*g* for 5 min, the supernatant was collected and used as the nuclear extract. Each step was performed on ice at 4 °C.

Immunoblotting analyses

Neurons were harvested after drug exposure, and the cell lysates were prepared as described before[8]. Protein samples were electrophoresed on 10%−12% SDS-PAGE gels. The antibodies used were as follows: rabbit polyclonal antibodies against pp38, p38, pERK, ERK, pJNK, JNK, pAkt, Akt, and H3 (1:1000; all from Cell Signaling Technology, Danvers, MA, USA), rabbit antibodies against pp85, p85, p65, and Bcl-2 (1:1000; all from Chemicon, Temecula, CA, USA), mouse antibody against β-actin (1:2000; Sigma, St Louis, MO, USA), and sheep antibody against Cyto *c* (1:1000; Chemicon, Temecula, CA, USA).

RNA extraction, RT-PCR and real-time PCR

Total RNA was extracted by using Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. RNA (2 μg) was reverse-transcribed by using the PrimeScript RT reagent kit (Takara, Japan), and single stranded cDNA was amplified by quantitative real-time PCR with the SYBR Premix Ex Taq Kit (Takara, Japan) on a Mastercycler ep realplex real-time PCR system (Eppendorf, Hamburg, Germany). The fold change was calculated using the ΔΔCt method ($2^{-\Delta\Delta Ct}$) with GAPDH as the internal control. The following primers were used: *Bcl-2*, 5′-CCCTGTGGATGACTGAGTAC-3′ and 5′-GCATGTTGACTTCACTTGTG-3′; *GAPDH*, 5′-CCACTCCTCCACCTTTGAC-3′ and 5′-ACCCTGTTGCTGTAGCCA-3′. Data are presented as the means±SEM of 4 independent experiments with 3 real-time PCR replicates.

Chromatin immunoprecipitation (ChIP) assay

ChIP assays were performed using a ChIP assay kit (Millipore, Billerica, MA, USA) according to the manufacturer's protocol. In brief, after drug treatment, neuronal cells were cross-linked with formaldehyde, lysed and sonicated. Immunoprecipitation was performed with antibodies to NF-κB (Cell Signaling Technology, Danvers, MA, USA), acetyl-H3 and acetyl-H4 (both from Chemicon, Temecula, CA, USA). Additionally, samples were immunoprecipitated with rabbit IgG as a negative control. Immunoprecipitated DNA was subjected to semi-quantitative and quantitative real-time PCR analyses using primers specific for the *Bcl-2* promoter (5′-TGTA-

ACTTTCAATGGACGC-3′ and 5′-ATCTACTTCCTCCG-CAATG-3′). After 30 cycles of amplification, 20 μL of the PCR product was analyzed on 2% agarose gels. Real-time PCR was performed as described above.

Statistical analysis

The data are shown as the mean±SEM and were subjected to statistical analysis using one-way ANOVA. The level of statistical significance was set at $P<0.05$.

Results

Prolonged DADLE exposure selectively activates the PI3K/Akt/NF-κB pathway in NaN_3-treated neurons

To examine the influence of prolonged DADLE stimulation on MAPK and Akt activity in NaN_3-treated neurons, primary rat cortical neuron cultures were treated with 80 μmol/L NaN_3 and DADLE or NTI for 2 d. The phosphorylation levels and the total levels of ERK, JNK, p38 and Akt were detected by immunoblotting after drug treatment. Neither the phosphorylation levels nor the total levels of ERK, p38 or JNK were affected by NaN_3 (Figure 1A); however, the levels of pAkt were reduced by NaN_3 (0.4-fold higher than those of the controls), and the total Akt levels were not affected (Figure 1C). DADLE not only reversed the NaN_3-induced inhibition of Akt activation but also further increased the levels of pAkt (1.72-fold higher than those of the controls); these effects were abolished by NTI (Figure 1C).

Next, the phosphorylation levels and total levels of the p85 subunit of PI3K were determined by immunoblotting after drug treatment. As shown in Figure 1B, the levels of pp85 were significantly decreased by NaN_3, and DADLE not only reversed the NaN_3-induced decrease in pp85 levels but also further elevated PI3K activity; these effects were abolished by NTI. Moreover, the total levels of p85 were not altered in any group after drug exposure. LY296004, a selective PI3K inhibitor, also prevented Akt activation in the neurons exposed to DADLE and NaN_3 (Figure 1C), indicating that PI3K acts as a downstream signal of DOR stimulation and is required for Akt activation in NaN_3-treated neurons.

To explore the impact of sustained DADLE stimulation on the activity of NF-κB in NaN_3-treated neurons, translocation of the p65 subunit of NF-κB into the nucleus was detected by immunoblotting after drug exposure. The levels of p65 in the nucleus were remarkably decreased by NaN_3, and this effect was attenuated by DADLE. The effect of DADLE was abolished by NTI, LY296004 and Akt inhibitor VIII, implying that DOR stimulation reverses NaN_3-induced NF-κB inhibition via the PI3K/Akt pathway.

The data above suggest that sustained DOR stimulation with DADLE selectively activates the PI3K/Akt/NF-κB pathway but not MAPKs in the NaN_3 model.

Prolonged DADLE treatment reduces cell injury in NaN_3-treated neurons via the PI3K/Akt pathway

To determine whether the protective effect of sustained DADLE stimulation is mediated by the PI3K/Akt pathway, neuronal damage was determined by measuring LDH release into the culture medium after drug exposure, and cell viability was detected by using a CCK-8 kit. DADLE completely suppressed the increase in LDH release induced by NaN_3 and restored cell viability; these effects were abolished by LY296004 and VIII (Figure 2). These data imply that sustained DOR stimulation reduces NaN_3-induced neuronal injury via the PI3K/Akt pathway.

Prolonged DADLE exposure enhances Bcl-2 expression and attenuates Cyto *c* release in NaN_3-treated neurons through the PI3K/Akt pathway

NaN_3 is a specific complex IV inhibitor widely used to induce mitochondrial dysfunction and Cyto *c* release[19, 20]. To explore the effect of sustained DADLE stimulation on mitochondrial Cyto *c* release in NaN_3-treated neurons, the levels of cytosolic Cyto *c* were tested by immunoblotting after drug exposure. As seen in Figure 3A, Cyto *c* levels in the cytosolic fraction were robustly increased by NaN_3; this effect was suppressed by DADLE. The effect of DADLE was totally abolished by NTI, LY296004 and VIII, implying that sustained DOR stimulation in NaN_3-treated neurons prevents Cyto *c* release through the PI3K/Akt pathway.

Bcl-2 is a well-known anti-apoptosis protein that prevents Cyto *c* release[21]. To investigate the impact of sustained DOR activation on Bcl-2 expression in NaN_3-exposed neurons, Bcl-2 levels were determined by immunoblotting after drug exposure. As shown in Figure 3B, Bcl-2 levels were considerably reduced by NaN_3, and this effect was reversed by DADLE; furthermore, the effect of DADLE was entirely prevented by NTI, LY296004 and VIII. Next, the mRNA levels of *Bcl-2* were determined after drug treatment. *Bcl-2* mRNA levels were significantly decreased by NaN_3; this effect was reversed by DADLE (Figure 3C). Again, the effect of DADLE was completely inhibited by NTI, LY296004 and VIII, indicating that sustained DOR stimulation with DADLE in NaN_3-treated neurons restores Bcl-2 protein levels by attenuating its transcriptional dysfunction via the PI3K/Akt pathway.

These results indicate that sustained DOR stimulation with DADLE decreases cell damage in NaN_3-treated neurons by promoting Bcl-2 expression and preventing Cyto *c* release through the PI3K/Akt pathway.

Prolonged DOR stimulation with DADLE enhances the NF-κB binding activity and histone acetylation of the *Bcl-2* promoter in neurons treated with NaN_3

NF-κB has been reported to promote the transcriptional activity of Bcl-2[22]; therefore, we further investigated whether the upregulation of Bcl-2 transcription is modulated by NF-κB during sustained DOR activation. According to ChIP assays, our results showed that the levels of NF-κB bound to the *Bcl-2* promoter were remarkably reduced by NaN_3; this effect was completely blocked by DADLE (Figure 4). The effect of DADLE was abolished by NTI, which indicates that sustained DOR activation enhanced NF-κB binding to the *Bcl-2* promoter

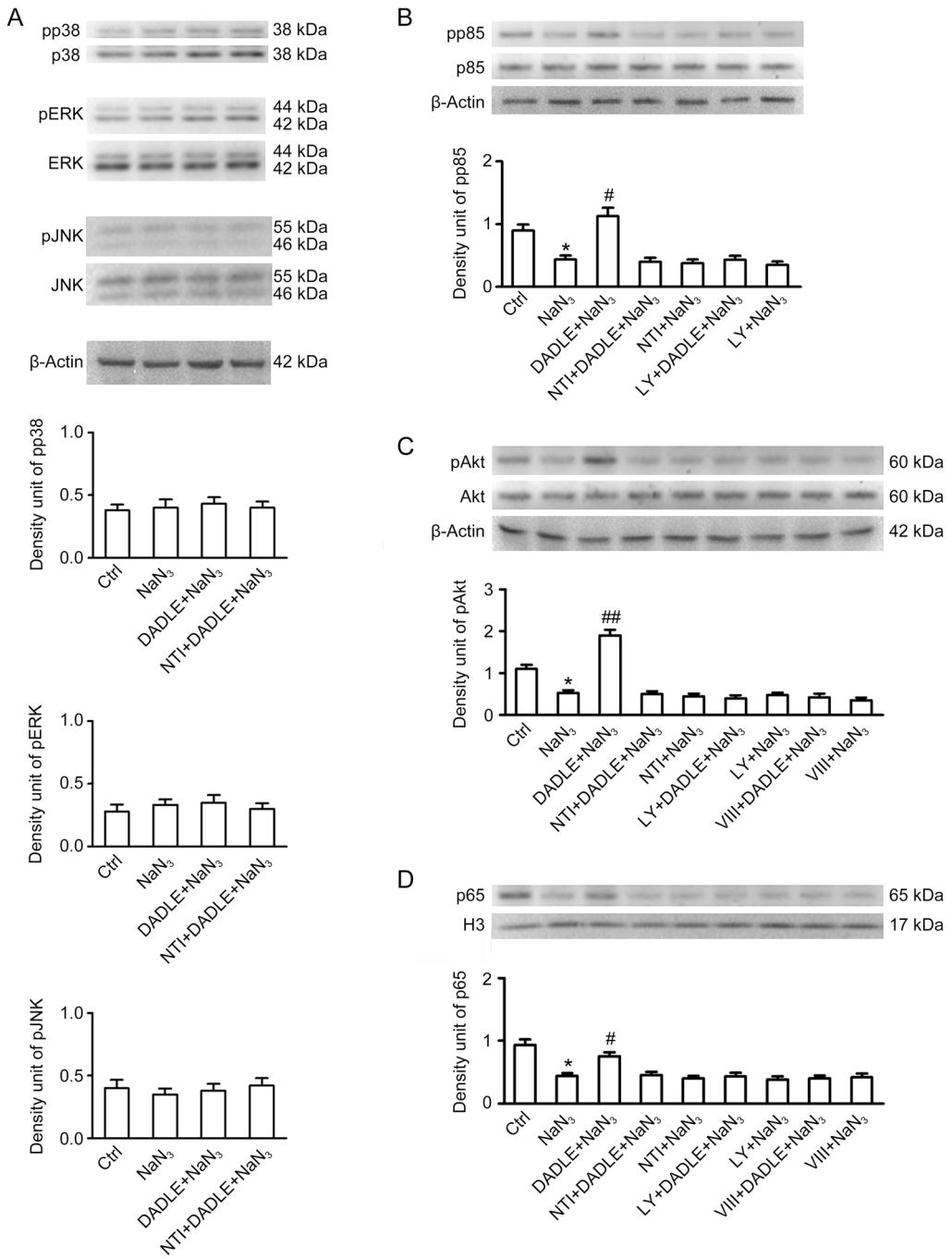

Figure 1. Sustained DOR stimulation with DADLE selectively activates the PI3K/Akt/NF-κB pathway in NaN_3-treated neurons. Primary rat cortical neuron cultures were treated with 80 μmol/L NaN_3 and with DADLE (0.1 μmol/L) or NTI (0.1 μmol/L) for 2 d to activate or inhibit DOR activity. The phosphorylation levels and total levels of ERK, JNK, p38, the p85 subunit of PI3K, Akt, and the p65 subunit of NF-κB in the nucleus were detected by immunoblotting after drug treatment. DADLE had no effect on the levels of phosphorylated p38 (pp38), phosphorylated ERK (pERK) or phosphorylated JNK (pJNK) (A) but further increased the phosphorylation levels of the regulatory p85 subunit of PI3K (pp85) (B) and Akt (pAkt) (C) and promoted the nuclear translocation of p65 (D) in neurons treated with NaN_3 for 2 d; these effects were abolished by the presence of NTI, LY296004 (PI3K inhibitor; 20 μmol/L) or Akt inhibitor VIII (5 μmol/L). $^{*}P<0.05$ compared to Ctrl; $^{\#}P<0.05$, $^{\#\#}P<0.01$ compared to the NaN_3 group. $n=5$.

in NaN_3-treated neurons.

ChIP was also used to determine the effect of DADLE on the histone acetylation level of the *Bcl-2* promoter in NaN_3-treated neurons. As shown in Figure 5, the levels of acetylated H3 and H4 for the *Bcl-2* promoter were both remarkably reduced by NaN_3; these effects were completely blocked by DADLE. The effects of DADLE were abolished by NTI. These data suggest that sustained DOR activation increased the histone acetylation levels of the *Bcl-2* promoter in neurons treated with NaN_3.

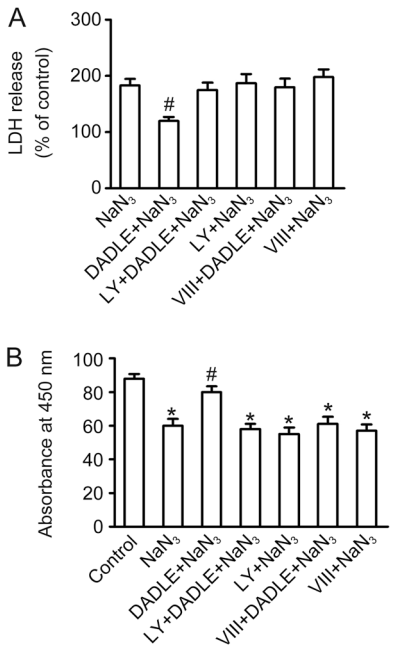

Figure 2. Sustained DOR stimulation with DADLE reduces cell injury in NaN_3-treated neurons via the PI3K/Akt pathway. Neuron cultures were treated with NaN_3 and DADLE, LY296004 or Akt inhibitor VIII. LDH release into the culture medium was measured after drug exposure for 2 d (A), and cell viability was tested with a CCK-8 kit (B). DADLE manifestly reduced the NaN_3-induced increase in LDH release and restored cell viability; these effects were reversed by co-treatment with LY296004 (20 μmol/L) or Akt inhibitor VIII (5 μmol/L). $^{*}P<0.05$ compared to Ctrl; $^{\#}P<0.05$ compared to the NaN_3 group. $n=5$.

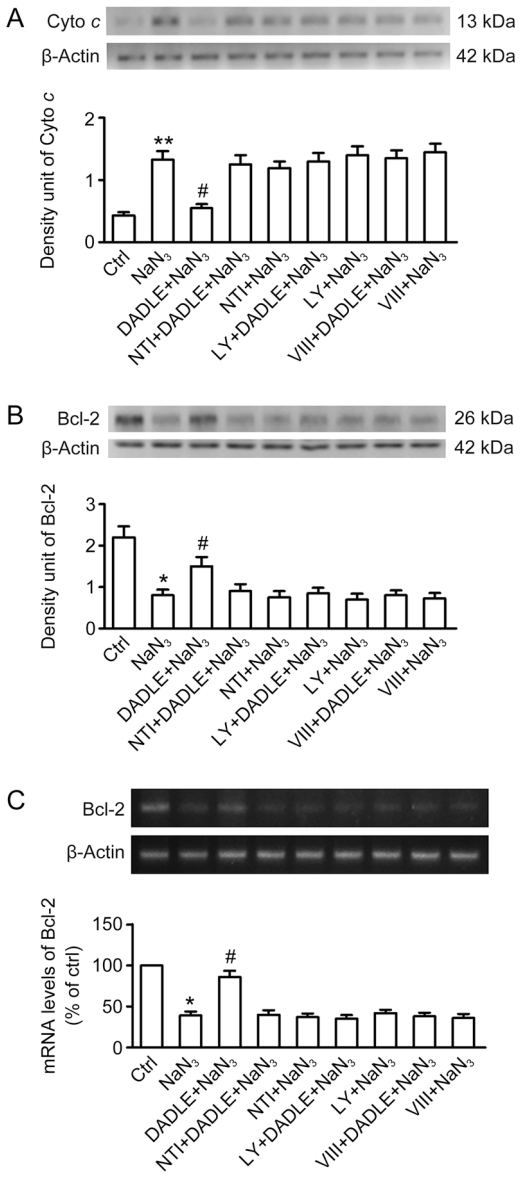

Figure 3. Sustained DOR activation with DADLE attenuates Cyto *c* release and enhances Bcl-2 expression in NaN_3-treated neurons through the PI3K/Akt pathway. Neuron cultures were treated with NaN_3 and DADLE, NTI, LY296004 or Akt inhibitor VIII for 2 d. The protein levels of cytosolic Cyto *c* (A) and Bcl-2 (B) in neurons were measured by immunoblotting after drug exposure, and the mRNA levels of *Bcl-2* were also determined (C). Cytosolic Cyto *c* levels were robustly increased in NaN_3-treated neurons, whereas the mRNA levels and protein levels of Bcl-2 were both remarkably decreased. DADLE suppressed the downregulation of Bcl-2 and the increase in cytosolic Cyto *c* levels; these effects were abolished by the co-administration of NTI, LY296004 or Akt inhibitor VIII. $^{*}P<0.05$, $^{**}P<0.01$ compared to Ctrl; $^{\#}P<0.05$ compared to the NaN_3 group. $n=5$.

Discussion

So far, there are no effective treatments to stop or reverse neurodegeneration after a stroke. Despite the encouraging results seen in preclinical studies, most cytoprotective drugs have failed in clinical trials. One of the main reasons for this is that those drugs showed protection at only the very early stage of neurodegeneration, and their effects did not last as the disease developed.

Our earlier work found that stimulating DOR with DADLE for both a short (minutes) and long (days) time resulted in significant neuroprotective effects in the NaN_3 model[8, 17]. These results imply that DADLE provides immediate protection at the early stage of neuronal injury and that its protective effects would last for days if the toxin is not removed. Our further investigations of the underlying mechanisms revealed that at least two downstream signaling pathways may be sequentially activated and responsible for the neuroprotective effects of DADLE against mitochondrial injury. While short-term DADLE stimulation increases neuronal survival through the PKC-mitochondrial ERK pathway[8], sustained DOR activation with DADLE in the present study had little effect on MAPK activity in NaN_3-treated neurons but selectively activated the PI3K/Akt/NF-κB pathway; these actions might mediate the neuroprotective effects of prolonged DOR activation.

Though the intricacies of the role of DOR in neuroprotection are not yet well-established, several endogenous neuroprotective pathways have been reported to be involved. Activating DOR via hypoxia preconditioning can balance the pERK and pp38 levels through the PKC/MAPK pathway; this action elicits neuroprotective effects in a severe hypoxia model *in vitro*[4]. Narita *et al* found that the neurogenesis and anti-apoptosis

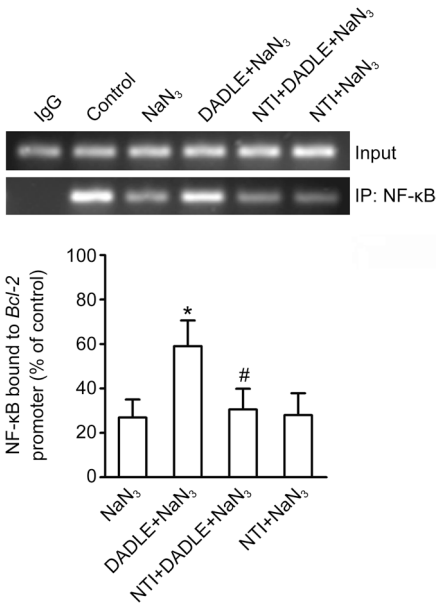

Figure 4. Sustained DOR activation with DADLE promotes NF-κB binding to the *Bcl-2* promoter in NaN_3-treated neurons. Neuron cultures were treated with NaN_3 and DADLE or NTI for 2 d, and chromatin immunoprecipitation (ChIP) analyses were performed with an antibody to NF-κB after drug treatment. DNA samples without immunoprecipitation served as the input. Representative semi-quantitative PCR analyses and quantitative real-time PCR analyses of the ChIP samples show that the levels of NF-κB bound to the *Bcl-2* promoter were remarkably reduced by NaN_3; this effect was completely blocked by DADLE. The effect of DADLE was abolished by NTI. $^{*}P<0.05$ compared to the NaN_3 group; $^{\#}P<0.05$ compared to the DADLE+NaN_3 group. $n=5$.

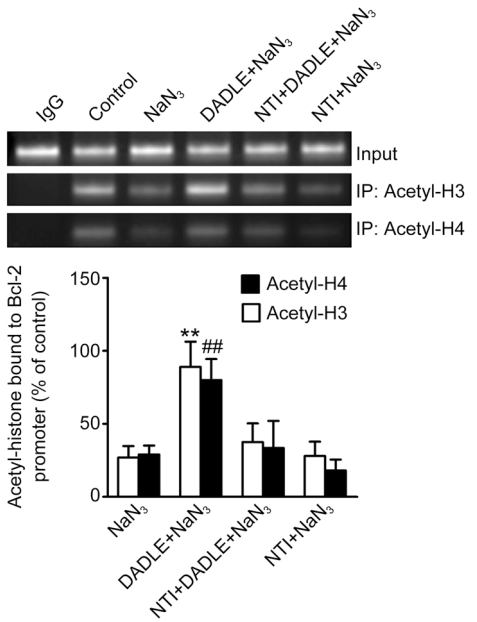

Figure 5. Sustained DOR stimulation with DADLE increased the histone acetylation levels of the *Bcl-2* promoter in NaN_3-treated neurons. Neuron cultures were exposed to NaN_3 and DADLE or NTI for 2 d, and chromatin immunoprecipitation (ChIP) analyses were performed with antibodies to acetyl-H3 or acetyl-H4 after drug treatment. Representative semi-quantitative PCR analyses and quantitative real-time PCR analyses of the ChIP samples show that the acetylation levels of both H3 and H4 for the *Bcl-2* promoter were decreased by NaN_3; these effects were both completely inhibited by DADLE. The effects of DADLE were totally reversed by co-treatment with NTI. $^{**}P<0.01$ compared to acetyl-H3 in the NaN_3 group; $^{\#\#}P<0.01$ compared to acetyl-H4 in the NaN_3 group. $n=5$.

effects of DOR stimulation may rely on Trk phosphorylation, which is associated with PI3K, MEK, and CaMKII activation in normally cultured neural stem cells and in H_2O_2-treated cells[23]. DOR activation also protects NG108-15 cells from pertussis toxin insult through activating the PI3K/Akt signaling pathway[15]. This evidence further indicates that DOR activation causes potent neuroprotection through distinct pathways, which makes DOR a potential therapeutic target.

The role of DOR in neuronal transcription and gene expression has not been well studied until recently. It has been reported that DADLE induces transcription inhibition, especially in apoptotic pathways, which may result in cellular metabolism arrest and provide neuroprotective effects[24, 25]. Nevertheless, this effect of DADLE is probably DOR-independent. Sen *et al* found that DADLE upregulates NGF by activating the PI3K/Akt/NF-κB pathway[26]. Our present work showed that DADLE upregulates Bcl-2 expression and exhibits anti-apoptotic effects, which are very likely mediated by DOR. These findings indicate that DADLE may bi-directionally regulate gene expression in neuronal cells; thus, the role of DOR remains unclear and needs further investigation.

This is the first study to demonstrate that prolonged DADLE exposure could epigenetically promote Bcl-2 expression in NaN_3-treated neurons. Bcl-2 is an important anti-apoptosis protein and is tightly associated with cell survival regulation. Evidence has shown that downregulating Bcl-2 via siRNA leads to increased apoptotic markers and ROS levels[27], whereas overexpressing Bcl-2 protects neurons from glutamate insult[28] and focal cerebral ischemia[9]. In our present study, we found that Bcl-2 levels were downregulated during NaN_3 exposure; this effect was reversed by the co-administration of DADLE. This change in Bcl-2 protein levels is consistent with that in mRNA levels and was inhibited by LY296004 and VIII, indicating that sustained DADLE stimulation may restore Bcl-2 protein levels mainly by attenuating its transcriptional dysfunction via PI3K/Akt signaling.

The anti-apoptotic functions of Bcl-2 are not only associated with its protein levels but also regulated by its phosphorylation. It has been suggested that in a mitotic arrest-induced model, multi-site phosphorylation of Bcl-2 within an unstructured loop inhibits its anti-apoptotic functions[29] . In contrast, the phosphorylation of Bcl-2 at S70 prevents apoptosis in cytokine-dependent cell lines[30]. Therefore, further studies are needed to determine how the phosphorylation status of Bcl-2 is affected by NaN_3 and DADLE treatment.

NF-κB is an important transcriptional factor with five different subunit isoforms that are ubiquitously expressed; NF-κB participates in the regulation of the expression of many

genes, and some of these genes are involved in the process of apoptosis[31]. Several studies showed that translocation of the NF-κB p65 subunit into the nucleus is activated via the PI3K/Akt pathway; there, it participates in regulating anti-apoptotic gene expression[32, 33], possibly through enhancing acetylation efficiency in the p65 binding region[34]. In the present study, we revealed that DOR stimulation reversed NaN_3-induced p65 inhibition via the PI3K/Akt pathway and promoted Bcl-2 transcription by increasing the histone acetylation of the *Bcl-2* promoter. These results are supported by other studies showing that NF-κB might directly bind to the *Bcl-2* promoter and promote its transcription[35, 36], as well as the fact that sustained DOR activation enhanced p65 binding to the *Bcl-2* promoter in neurons treated with NaN_3 in this study. Therefore, upon sustained DADLE stimulation in NaN_3-induced mitochondrial injury, the epigenetic upregulation of Bcl-2 expression might play an important role in the downstream signaling of DOR neuroprotection. Since Bcl-2 levels could be affected by both the expression and degradation processes, whether NaN_3 or DADLE affect Bcl-2 protein degradation still needs further investigation.

In conclusion, we showed here that the mechanism underlying sustained DOR activation by DADLE is very different from the mechanism underlying its short-term stimulation in NaN_3-treated neurons. A different signaling pathway (PI3K/Akt/NF-κB) is activated, and it further promotes Bcl-2 expression through epigenetic regulation. Bcl-2 upregulation suppresses Cyto *c* release and rescues neuronal cells (Figure 6). The present work is an essential supplement to our earlier studies and implies that DADLE may provide a distinct therapeutic opportunity for stroke treatment.

Acknowledgements

This work was supported by the Science and Technology Commission Program of Shanghai (No 06DZ1734) and the National Natural Science Foundation of China (No 30873318).

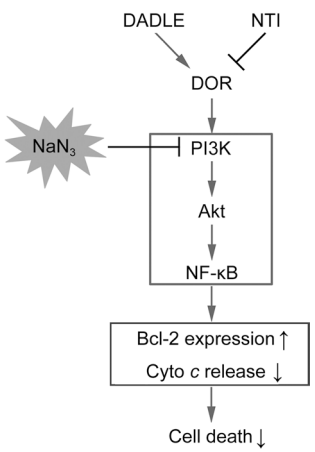

Figure 6. Scheme showing the pathways that mediate the neuroprotective effects of sustained DOR stimulation with DADLE against NaN_3 insult.

Author contribution

Jing-chun GUO and Cui-qing ZHU designed the study; Min ZHU and Ming LIU performed the research; Min ZHU and Qi-lin GUO analyzed the data; and Min ZHU, Ming LIU and Jing-chun GUO wrote the paper.

References

1 Zhang J, Haddad GG, Xia Y. delta-, but not mu- and kappa-, opioid receptor activation protects neocortical neurons from glutamate-induced excitotoxic injury. Brain Res 2000; 885: 143–53.
2 Law PY, Wong YH, Loh HH. Molecular mechanisms and regulation of opioid receptor signaling. Annu Rev Pharmacol Toxicol 2000; 40: 389–430.
3 Zhang J, Qian H, Zhao P, Hong SS, Xia Y. Rapid hypoxia preconditioning protects cortical neurons from glutamate toxicity through delta-opioid receptor. Stroke 2006; 37: 1094–9.
4 Ma MC, Qian H, Ghassemi F, Zhao P, Xia Y. Oxygen-sensitive {delta}-opioid receptor-regulated survival and death signals: novel insights into neuronal preconditioning and protection. J Biol Chem 2005; 280: 16208–18.
5 Zhang J, Gibney GT, Zhao P, Xia Y. Neuroprotective role of delta-opioid receptors in cortical neurons. Am J Physiol Cell Physiol 2002; 282: C1225–34.
6 Drolet G, Dumont EC, Gosselin I, Kinkead R, Laforest S, Trottier JF. Role of endogenous opioid system in the regulation of the stress response. Prog Neuropsychopharmacol Biol Psychiatry 2001; 25: 729–41.
7 Peng PH, Huang HS, Lee YJ, Chen YS, Ma MC. Novel role for the delta-opioid receptor in hypoxic preconditioning in rat retinas. J Neurochem 2009; 108: 741–54.
8 Zhu M, Li M, Yang F, Ou X, Ren Q, Gao H, *et al*. Mitochondrial ERK plays a key role in delta-opioid receptor neuroprotection against acute mitochondrial dysfunction. Neurochem Int 2011; 59: 739–48.
9 Narita M, Kuzumaki N, Miyatake M, Sato F, Wachi H, Seyama Y, *et al*. Role of delta-opioid receptor function in neurogenesis and neuroprotection. J Neurochem 2006; 97: 1494–505.
10 Chao D, Balboni G, Lazarus LH, Salvadori S, Xia Y. Na^+ mechanism of delta-opioid receptor induced protection from anoxic K^+ leakage in the cortex. Cell Mol Life Sci 2009; 66: 1105–15.
11 Chao D, Bazzy-Asaad A, Balboni G, Salvadori S, Xia Y. Activation of DOR attenuates anoxic K^+ derangement via inhibition of Na^+ entry in mouse cortex. Cereb Cortex 2008; 18: 2217–27.
12 Chao D, Donnelly DF, Feng Y, Bazzy-Asaad A, Xia Y. Cortical delta-opioid receptors potentiate K^+ homeostasis during anoxia and oxygen-glucose deprivation. J Cereb Blood Flow Metab 2007; 27: 356–68.
13 Chao D, Bazzy-Asaad A, Balboni G, Xia Y. delta-, but not mu-, opioid receptor stabilizes K^+ homeostasis by reducing Ca^{2+} influx in the cortex during acute hypoxia. J Cell Physiol 2007; 212: 60–7.
14 Ke S, Dian-san S, Xiang-rui W. Delta opioid agonist [D-Ala2, D-Leu5] enkephalin (DADLE) reduced oxygen-glucose deprivation caused neuronal injury through the MAPK pathway. Brain Res 2009; 1292: 100–6.
15 Heiss A, Ammer H, Eisinger DA. delta-Opioid receptor-stimulated Akt signaling in neuroblastoma x glioma (NG108-15) hybrid cells involves receptor tyrosine kinase-mediated PI3K activation. Exp Cell Res 2009; 315: 2115–25.
16 Lv MR, Li B, Wang MG, Meng FG, Yu JJ, Guo F, *et al*. Activation of the PI3K-Akt pathway promotes neuroprotection of the delta-opioid receptor agonist against cerebral ischemia-reperfusion injury in rat models. Biomed Pharmacother 2017; 93: 230–7.

17 Zhu M, Li MW, Tian XS, Ou XM, Zhu CQ, Guo JC. Neuroprotective role of delta-opioid receptors against mitochondrial respiratory chain injury. Brain Res 2009; 1252: 183–91.
18 Zhu L, Derijard B, Chakrabandhu K, Wang BS, Chen HZ, Hueber AO. Synergism of PI3K/Akt inhibition and Fas activation on colon cancer cell death. Cancer Lett 2014; 354: 355–64.
19 Safiulina D, Veksler V, Zharkovsky A, Kaasik A. Loss of mitochondrial membrane potential is associated with increase in mitochondrial volume: physiological role in neurones. J Cell Physiol 2006; 206: 347–53.
20 Marino S, Marani L, Nazzaro C, Beani L, Siniscalchi A. Mechanisms of sodium azide-induced changes in intracellular calcium concentration in rat primary cortical neurons. Neurotoxicology 2007; 28: 622–9.
21 Jiang X, Wang X. Cytochrome *c*-mediated apoptosis. Annu Rev Biochem 2004; 73: 87–106.
22 Barkett M, Gilmore TD. Control of apoptosis by Rel/NF-kappaB transcription factors. Oncogene 1999; 18: 6910–24.
23 Jeong S, Kim SJ, Jeong C, Lee S, Jeong H, Lee J*, et al*. Neuroprotective effects of remifentanil against transient focal cerebral ischemia in rats. J Neurosurg Anesthesiol 2012; 24: 51–7.
24 Tian J, Gu Y, Sun K, Wang B, Chen J, Wang X*, et al*. [D-Ala2, D-Leu5] encephalin (DADLE) reversibly inhibits cellular transcription in neurons without causing cell injury. Brain Res 2014; 1565: 1–7.
25 Wang Z, Tang B, Tang F, Li Y, Zhang G, Zhong L*, et al*. Protection of rat intestinal epithelial cells from ischemia/reperfusion injury by (D-Ala2, D-Leu5)-enkephalin through inhibition of the MKK7-JNK signaling pathway. Mol Med Rep 2015; 12: 4079–88.
26 Sen D, Huchital M, Chen YL. Crosstalk between delta opioid receptor and nerve growth factor signaling modulates neuroprotection and differentiation in rodent cell models. Int J Mol Sci 2013; 14: 21114–39.
27 Kesaraju S, Nayak G, Prentice HM, Milton SL. Upregulation of Hsp72 mediates anoxia/reoxygenation neuroprotection in the freshwater turtle via modulation of ROS. Brain Res 2014; 1582: 247–56.
28 Wang Q, Chao D, Chen T, Sandhu H, Xia Y. delta-Opioid receptors and inflammatory cytokines in hypoxia: differential regulation between glial and neuron-like cells. Transl Stroke Res 2014; 5: 476–83.
29 Bassik MC, Scorrano L, Oakes SA, Pozzan T, Korsmeyer SJ. Phosphorylation of BCL-2 regulates ER Ca^{2+} homeostasis and apoptosis. EMBO J 2004; 23: 1207–16.
30 Ito T, Deng X, Carr B, May WS. Bcl-2 phosphorylation required for anti-apoptosis function. J Biol Chem 1997; 272: 11671–3.
31 Baeuerle PA, Henkel T. Function and activation of NF-kappa B in the immune system. Annu Rev Immunol 1994; 12: 141–79.
32 Li G, Kalabis J, Xu X, Meier F, Oka M, Bogenrieder T*, et al*. Reciprocal regulation of MelCAM and AKT in human melanoma. Oncogene 2003; 22: 6891–9.
33 Dhawan P, Singh AB, Ellis DL, Richmond A. Constitutive activation of Akt/protein kinase B in melanoma leads to up-regulation of nuclear factor-kappaB and tumor progression. Cancer Res 2002; 62: 7335–42.
34 Lanzillotta A, Sarnico I, Ingrassia R, Boroni F, Branca C, Benarese M*, et al*. The acetylation of RelA in Lys310 dictates the NF-kappaB-dependent response in post-ischemic injury. Cell Death Dis 2010; 1: e96.
35 Huang X, Qin J, Lu S. Kanglaite stimulates anticancer immune responses and inhibits HepG2 cell transplantation induced tumor growth. Mol Med Rep 2014; 10: 2153–9.
36 Shu G, Yang T, Wang C, Su H, Xiang M. Gastrodin stimulates anticancer immune response and represses transplanted H22 hepatic ascitic tumor cell growth: Involvement of NF-kappaB signaling activation in $CD4^+$ T cells. Toxicol Appl Pharmacol 2013; 269: 270–9.