Estrogen-related receptor α (ERRα) functions in the hypoxic injury of microglial cells

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Abstract

Introduction: Hypoxia is a common pathological condition after spinal cord injury. Oestrogen-related receptor alpha (ERRα), as a key regulator of energy metabolism and mitochondrial functions, plays an important role in maintaining cell homeostasis. However, its role in hypoxic spinal microglia has not been fully elaborated. This study investigated the receptor's activity when these cells are hypoxic and used as an *in vitro* model. **Material and Methods:** In this study, microglia (BV2) were exposed to cobalt chloride as a hypoxic model, and the inverse agonist of ERRα, XCT790, and pyrido[1,2-α]-pyrimidin-4-one were used to regulate the expression of the receptor to explore the ERRα-related mechanisms involved in hypoxic spinal cord injury (SCI). **Results:** ERRα promoted autophagy in BV2 cells and inhibited the activation of the p38 mitogen-activated protein kinase (MAPK) pathway and the expression of anti-inflammatory factors under hypoxic conditions. It also promoted the expression of fibronectin type III domain containing protein 5 (FNDC5). **Conclusion:** When a hypoxic SCI occurs, ERRα may maintain the homeostasis of spinal cord nerve cells by regulating autophagy and the p38MAPK/nuclear factor-kappa B cell and FNDC5/brain-derived neurotrophic factor signalling pathways, which are beneficial to the recovery of these cells.

Keywords: spinal cord injury, microglia, oestrogen-related receptor alpha, cobalt chloride, nuclear factor-kappa B.

Introduction

Spinal cord injury (SCI) is a neurological disorder that results from the destruction of the spinal cord's structure and function, resulting in neurological disorders and leading to the weakening or loss of voluntary skeletal muscle movement. It may lead to local tissue damage, ischaemia and hypoxia. Neurons in the central nervous system are extremely sensitive to changes in the internal environment. Hypoxia therefore not only causes neuronal degeneration and necrosis (9), but also aggravates the inflammatory response in the spinal cord.

Microglia, myeloid-derived macrophage-like cells, are the resident immune cells of the central nervous system (19). They maintain immune homeostasis through phenotype remodelling (M1 and M2 phenotypes), which is a highly energy-dependent process. During hypoxic injury, microglia are activated and begin different degrees of aggregation and proliferation in the area of injury (2). Morphologically, the microglia transition from ramified to amoeboid. Functionally, they migrate and phagocytose, helping to clear cellular debris. These cells also excrete significant amounts of interleukin (IL)-1β, IL-6 and tumour necrosis factor alpha (TNF-α) proinflammatory cytokines, as well as chemokines and oxidative metabolites, further exacerbating spinal cord injuries (1). They stimulate peripheral immune cells to infiltrate into the damaged area, leading to secondary spinal cord injuries.

During SCI, microglial cells constantly change and adjust their state to exert their immune-related effects. At the same time, the organismal metabolism adjusts to a state more suitable for immune cells, providing them with the necessary energy. Oestrogen-related receptor alpha (ERRα) is a key metabolic regulator that controls many nuclear-encoded mitochondrial enzymes involved in energy metabolism and mitochondrial biogenesis. Its deletion leads to an imbalanced antioxidative stress capacity in the body and induces the activation of microglial cells (23). Hypoxic injury causes microglial cells to alter their phenotype (6); and having in mind that

ERRα plays an important role in energy metabolism and mitochondrial functions in the body, we hypothesised that in hypoxia, ERRα likely is in the forefront in maintaining the homeostasis of spinal cord nerve cells.

In this study, we used BV2 microglial cells to establish an *in vitro* hypoxic model, which we used to clarify the role of ERRα in spinal microglial hypoxic injury.

Material and Methods

Cell culture. Immortalised murine BV2 microglial cells were maintained at 37°C in a 5% CO_2 atmosphere in Dulbecco's modified Eagle's medium supplemented with 10% (*v/v*) foetal bovine serum FBS, penicillin (100 U/mol) and streptomycin (100 μg/mL). The cells were first plated in a medium containing 2% FBS, and the following day the treatments began. Cells were treated daily for 2 days with 1 μM XCT790 (catalogue no. X4753; Sigma, China), the inverse agonist ligand of ERRα, and then treated with or without 100 μM $CoCl_2$ for 8 h. For overexpression studies, 3×10^6 BV2 cells were seeded onto 6-well plates containing 2% FBS, and the next day the cells were transfected with 30 μM of pyrido[1,2-α]-pyrimidin-4-one synthesised as previously described (17) for 24 h and treated with or without 100 μM $CoCl_2$ for 8 h. This the ERRα agonist treatment. To generate hypoxic stress conditions, one group of cells was only treated with 100 μM $CoCl_2$ for 8 h.

Measurement of cell viability using the cholecystokinin octapeptide (CCK8) assay. BV2 cells were seeded in 96-well plates at a density of 2×10^4 cells/well. Addition of $CoCl_2$ followed at different concentrations, and after culturing for 8 h, 10 μL of the CCK8 reagent was added to each well. The plates were incubated for 1.5 h at 37°C. Absorbance was measured with a Model 680 microplate reader (Bio-Rad Laboratories, Hercules, CA, USA) at 450 nm. Cytotoxicity was analysed according to the following formula:

cell viability (%) = [(Abs sample) − (Abs blank)] / [(Abs negative control) − (Abs blank)] × 100.

Abs sampl: Contains cell culture medium, CCK-8, $CoCl_2$,

Abs blank: Contains cell culture medium, CCK-8, $CoCl_2$,

Abs negative control: Cell-free medium, CCK-8, $CoCl_2$.

Wound-healing assay. When the BV2 cell density was greater than 80%, a linear wound was made by scraping a non-opening Pasteur pipette across the confluent cell layer. Cells were washed twice to remove detached cells and debris, and the cells were then treated independently with XCT790, pyrido[1,2-α]-pyrimidin-4-one and $CoCl_2$. The sizes of the wounds were observed and measured at specified intervals.

Wound healing rate (n h) = [Migration area (0 h) − Migration area (n h)] / Migration area (0 h)] × 100.

Western blotting. Western blot procedures were performed in accordance with a previously published experimental protocol (13). Membranes were blotted with primary and secondary antibodies as shown in Table 1, and all were from Proteintech (Rosemont, IL, USA) except for those against P-ERK1/2 and ERK1/2, which were products of Cell Signaling Technology (Danvers, MA, USA).

Reverse transcription quantitative-PCR (RT-qPCR). Total RNAs was extracted using TRIzol® reagent (Invitrogen, Waltham, MA, USA) The RNAs was reverse-transcribed using a RT Reagent Kit with gDNA Eraser (TaKaRa, Kusatsu, Japan). The protocol used SYBR Premix Ex Taq (TaKaRa) for a routine PCR, and the IQ SYBR Green Supermix (Bio-Rad) for a quantitative real-time PCR. The primers used are shown in Table 2. The specificity of the amplified PCR products was assessed using a melting curve analysis, and the results were calculated using the $2^{-\Delta\Delta Ct}$ method.

Table 1. Primary and secondary antibodies used for Western blotting

	Antibody	Dilution	Catalogue number
Primary	Beclin1	1:1,000	11306-1-AP
	LC3	1:1,000	14600-1-AP
	BDNF	1:1,000	28205-1-AP
	FNDC5	1:1,000	23995-1-AP
	p-p65	1:1,000	19771-1-AP
	p65	1:2,000	10745-1-AP
	p-p38	1:1,000	19771-1-AP
	p38	1:1,000	14064-1-AP
	IκB-α	1:1,000	10268-1-AP
	P-ERK1/2	1:1,000	9101
	ERK1/2	1:1,000	4695S
	ERRα	1:1,000	13826
	Tubulin	1:15,000	11224-1-AP
	β-actin	1:15,000	60008-1-lg
Secondary	HRP-conjugated Affinipure Goat Anti-Mouse IgG (H+L)	1:8,000	SA00001-1
	HRP-conjugated Affinipure Goat Anti-Rabbit IgG (H+L)	1:8,000	SA00001-2

LC3 – light chain 3; BDNF – brain-derived neurotrophic factor; FNDC5 – fibronectin type III domain containing protein 5; IκB-α – inhibitor of nuclear factor of kappa light polypeptide gene enhancer in B-cells, alpha; P-ERK1/2 – phosphor-ERK1/2; ERK1/2 – extracellular signal–regulated kinase 1/2; ERRα – oestrogen-related receptor alpha; HRP – horseradish peroxidase

Table 2. Primers used for protein amplification

Protein	Forward primer	Reverse primer
IL-4	5′-GGTCTCAACCCCCAGCTAGT-3′	5′-GCCGATGATCTCTCTCAAGTGAT-3′
IL-10	5′-GCTCTTACTGACTGGCATGAG-3′	5′-CGCAGCTCTAGGAGCAT GTG-3′
IL-6	5′-CCGGAGAGGAGACTTCACAG-3′	5′-GGAAAT TGGGGTAGGAAGGA-3′
TNF-α	5′-TACTGAACTTCGGGGTGAT TGGTCC-3′	5′-CAGCCTTGTCCCTTGAAGAGAAC-3′
P65	5′-GTATTGCTG TGCCTACCCGAAAC-3′	5′-GTTTGAGATCTGCCCTGATGGTAA-3′
P62	5'-GTTATGGCGTCGTTCACGGT-3′	5′-TCACAATGGTGGAGGGTGC-3′
HIF-1 α	5′-GATGGGGTTATGAGCCGGAAGA-3′	5′-CTGTGGCTGGGAGTTCTTC G-3′
β-actin	5′-CGTTGACATCCGTAAAGACC-3′	5′-AACAGTCCGC CTAGAAGCAC-3′

IL-4 – interleukin 4; *IL-10* – interleukin 10; *IL-6* – interleukin 6; *TNF-α* – tumour necrosis factor alpha; *HIF-1α* – hypoxic inducible factor 1-alpha

Immunofluorescence. The BV2 cells to be examined were grown on 18-mm diameter glass coverslips (Thermo Fisher Scientific, Pittsburgh, PA, USA), fixed with paraformaldehyde, washed three times with phosphate-buffered saline and blocked with 3% bovine serum albumin for 30 min. The cells were incubated with the corresponding primary antibody in blocking buffer overnight at 4°C, and next incubated again with a fluorescein isothiocyanate-conjugated anti-rabbit IgG secondary antibody (Sigma-Aldrich, St. Louis, MO, USA). They were then mounted in anti-fade solution (Molecular Probes Inc., Eugene, OR, USA). The slides were examined using an FV 1000 confocal laser scanning microscope (Olympus Corporation, Tokyo, Japan) equipped with a 100 × objective. Images were obtained using FluoView software (Olympus Corporation).

Statistical analysis. All experiments were performed three times. Comparisons of data between groups were made using one-way ANOVA. The data were visualised using GraphPad Prism 7 software (GraphPad Software, San Diego, CA, USA) and expressed as mean ± standard deviation. Values of $P < 0.05$ were considered significant.

Results

Effects of different CoCl₂ concentrations on cell viability. The BV2 cell survival rate after the 8 h treatment decreased as the CoCl₂ concentration increased. The cell survival rates were >90.00%, 78.82% and 42.01% when the CoCl₂ concentrations were 50–200 μmol/L, 300 μmol/L and 800 μmol/L (P<0.01), respectively (Fig. 1).

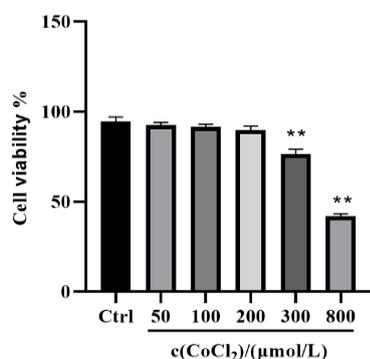**Fig. 1.** The effects of CoCl₂ on BV2 cell activity. Values represent the mean ± standard deviation for three independent experiments. **P<0.01

CoCl₂-induced hypoxic-inducible factor-1alpha (HIF-1α) expression. To observe the relationship between the expression of the hypoxia inducer HIF-1α and the hypoxia treatment time during hypoxic injury, BV2 cells were treated with 100 μmol/L CoCl₂ at the 0, 4, 8 and 12 h time points, and HIF-1α protein expression was detected using laser confocal technology.

The change in HIF-1α protein expression is shown in Fig. 2. The expression of HIF-1α at the protein level increased significantly during hypoxic injury in a time-dependent manner. Compared with the control group, the expression of HIF-1α was significantly higher after 4 h of the hypoxic treatment and continued to intensify after 8 h of treatment. The HIF-1α protein entered the nucleus from the cytoplasm. After 12 h of hypoxic treatment, the expression of HIF-1α gradually weakened.
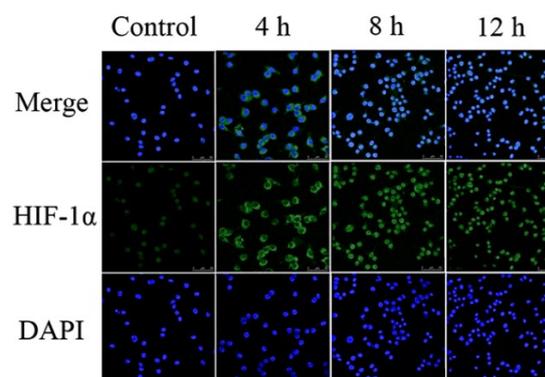

Fig. 2. The effects of different CoCl₂ treatment times on the expression of hypoxic inducible factors. The nucleus is indicated with blue fluorescence (4′,6-diamidino-2-phenylindole – DAPI) and the hypoxic-inducible factor 1-alpha (HIF-1α) protein is indicated with green fluorescence. Scale bar: 50 μm

Effects of XCT790 and pyrido[1,2-α]-pyrimidin-4-one on ERRα protein expression in BV2 cells. The change in ERRα protein expression is shown in Fig. 3. Compared with the control group, the expression level of ERRα in the ERRα agonist group was significantly greater, while its expression level in the XCT790 group was significantly lower (P<0.01).

The RT-q PCR results are shown Fig. 4. Compared with the control group, there was underexpression of HIF-1α in the cells treated with XCT790 to a significantly different degree (P <0.01), while there was overexpression in the cells treated with the ERRα agonist. Compared with the expression level of HIF-1α in the ERRα agonist + CoCl₂ group, the level in the cells treated with XCT790 + CoCl₂ had significantly decreased.
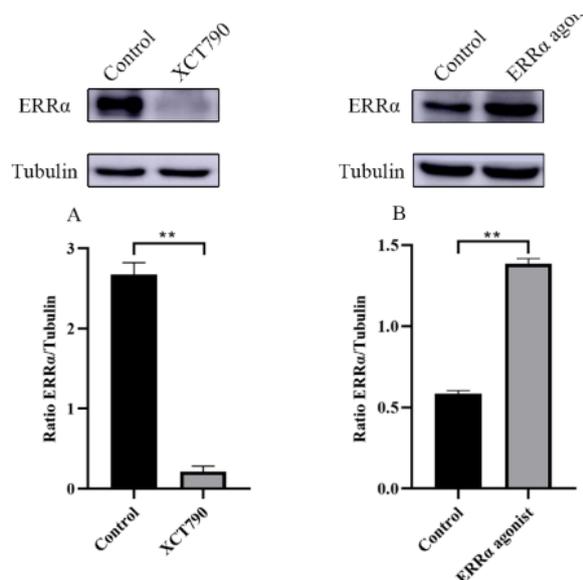
Fig. 3. Expression of ERRα in BV2 cells. A – Expression of ERRα in the control and XCT790 groups; B – Expression of ERRα in the control and ERRα agonist groups. Values represent the mean ± standard deviation for three independent experiments. **P<0.01
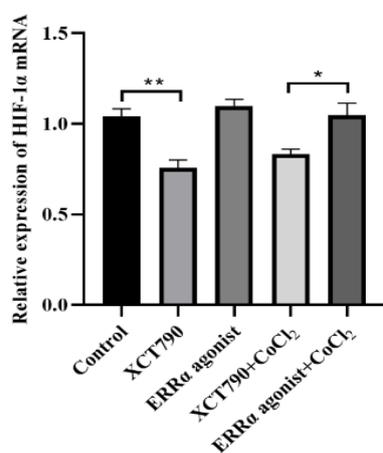

Fig. 4. The changes in hypoxic-inducible factor 1-alpha (HIF-1α) mRNA levels in BV2 cells after corresponding treatments. Values represent the mean ± standard deviation for three independent experiments. *P < 0.05; **P < 0.01

Effect of ERRα on autophagy in BV2 cells under hypoxic conditions. To clarify the role of ERRα in autophagy in hypoxia-damaged BV2 cells, the expression of autophagy-related light chain 3 (LC3) and Beclin1 proteins was detected using Western blotting, and the mRNA expression of autophagy-related gene p62 was assessed by RT-qPCR.

As shown in Fig. 5, the expression levels of Beclin1 and LC3 in the cells treated with CoCl$_2$ and XCT790 were significantly higher compared with the control group (P<0.01). In the ERRα agonist group, the expression levels of Beclin1 and LC3 had respectively decreased significantly (P<0.01) from and increased significantly (P<0.01), compared with the control group. When the CoCl$_2$ group was the standard for comparison of Beclin-1 and LC3 expression, the cells treated with XCT790+CoCl$_2$ expressed significantly more of both

(P<0.01), while the ERRα agonist group expressed significantly less of the former protein (P<0.01), and no significantly different amount of the second.

The results of the RT-qPCR experiment are shown in Fig. 5. Expression of p62 was upregulated over that of the control group in the cells treated with XCT790, and statistical significance applied (P<0.01). Expression of this protein was downregulated from that of the control group in the cells treated with ERRα agonist, and statistical significance also applied to this (P<0.01). The p62 protein was produced more intensively and significantly differently so by the cells treated with XCT790+CoCl$_2$ (P<0.01) seen against the production of this protein by cells of the ERRα agonist + CoCl$_2$ group.

Effect of ERRα on the activation of the p38MAPK pathway and the expression of anti-inflammatory factors in BV2 cells under hypoxic conditions. To explore the regulatory effects of ERRα on inflammatory cytokines in the hypoxic injury of BV2 cells, the expression levels of inhibitor of nuclear factor of kappa light polypeptide gene enhancer in B-cells, alpha (IκB-α), p38 and p65 were detected using Western blotting, and the mRNA expression levels of the IL-6, TNF-α, p65, IL-4 and IL-10 autophagy-related genes were assessed by qRT-PCR.

As shown in Fig. 6, compared with the control group, the expression levels of IκB-α in the cells treated with CoCl$_2$ decreased significantly (P < 0.01), while the expression levels of p38 and p65 increased significantly (P<0.01). The expression levels of p38 and p65 in the cells treated with XCT790 increased significantly (P<0.01), and the expression level of IκB-α was not significantly affected. In the ERRα agonist group, p38 and p65 showed significant respective augmentation and diminution of expression (P<0.01), while IκB-α did not demonstrate any significant effect on its expression. Compared with the CoCl$_2$ group, intensified expression of IκB-α and p65 was observed by the cells treated with XCT790+CoCl$_2$ (significant at P<0.01), but diminished expression of p38 was noted by these cells (also significant at P<0.01). The expression level of p38 in the cells treated with ERRα agonist+CoCl$_2$ significantly decreased (P<0.01), the expression level of p65 significantly increased (P<0.01), but the expression level of IκB-α was not significantly changed.

The results of the RT-qPCR experiment are shown in Fig. 7. The secretion of IL-6, TNF-α, p65 and IL-10 by the cells treated with XCT790 significantly surpassed that of the control group (P<0.01), while that of IL-4 did not match it by a significant margin (P<0.01). The concentration of IL-6 in the ERRα agonist group cells was not significantly different (P<0.01), but those of TNF-α, p65, IL-4, and IL-10 were significantly lower (P<0.01). IL-6, TNF-α, p65 and IL-10 were significantly more abundant (P<0.01) in the cells treated with XCT790+CoCl$_2$ than in those of the ERRα agonist+CoCl$_2$ group while IL-4 was significantly less so (P<0.01).
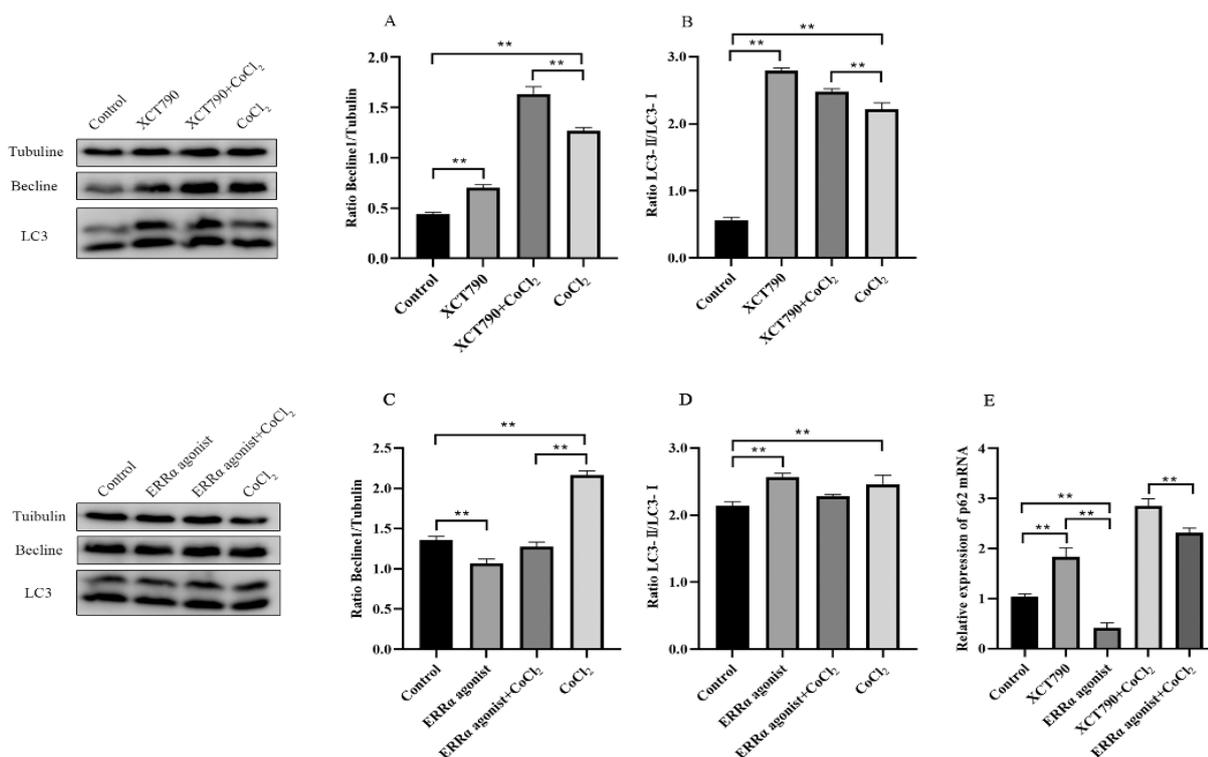
Fig. 5. Expression of Beclin1 and light chain 3 (LC3) in BV2 cells. A – Expression of Beclin1 in the control, XCT790, XCT790+CoCl$_2$ and CoCl$_2$ groups; B – The light chain (LC)3-II/LC3-I ratio in the control, XCT790, XCT790+CoCl$_2$ and CoCl$_2$ groups; C – Expression of Beclin1 in the control, oestrogen-related receptor alpha (ERRα) agonist, ERRα agonist+CoCl$_2$ and CoCl$_2$ groups; D – The LC3-II/LC3-I ratio in the control, ERRα agonist, ERRα agonist+CoCl$_2$ and CoCl$_2$ groups; E – The changes in p62 mRNA levels in BV2 cells after corresponding treatments. Values represent the mean ± standard deviation for three independent experiments. **$P<0.01$
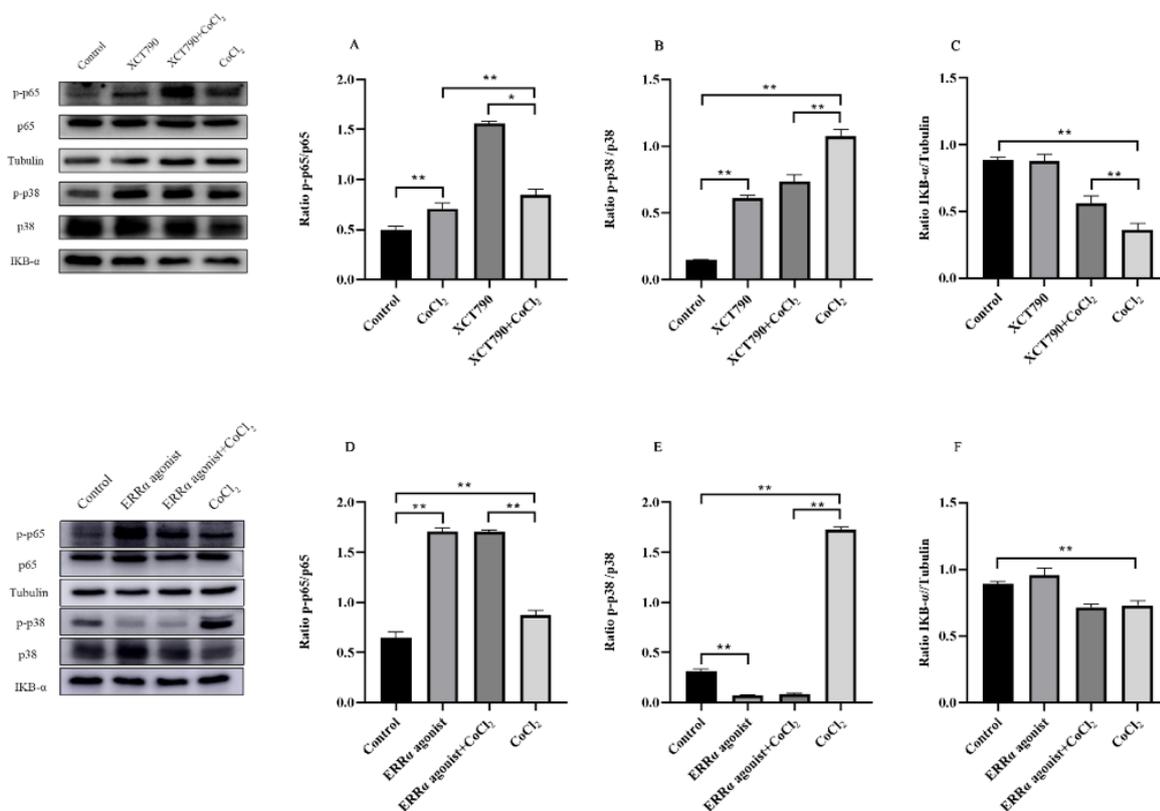

Fig. 6. Expression of p65, p38 and inhibitor of nuclear factor of kappa light polypeptide gene enhancer in B-cells, alpha (IκB-α) in BV2 cells. A – The p-p65/p65 ratio in the control, XCT790, XCT790+CoCl$_2$ and CoCl$_2$ groups; B – The p-p38/p38 ratio in the control, XCT790, XCT790+CoCl$_2$ and CoCl$_2$ groups; C – Expression of IκB-α in the control, XCT790, XCT790+CoCl$_2$ and CoCl$_2$ groups; D – The p-p65/p65 ratio in the control, oestrogen-related receptor alpha (ERRα) agonist, ERRα agonist+CoCl$_2$ and CoCl$_2$ groups; E – The p-p38/p38 ratio in the control, ERRα agonist, ERRα agonist+CoCl$_2$ and CoCl$_2$ groups; F – Expression of IκB-α in the control, ERRα agonist, ERRα agonist+CoCl$_2$ and CoCl$_2$ groups. Values are expressed as means ± standard deviation. *$P < 0.05$; **$P < 0.01$
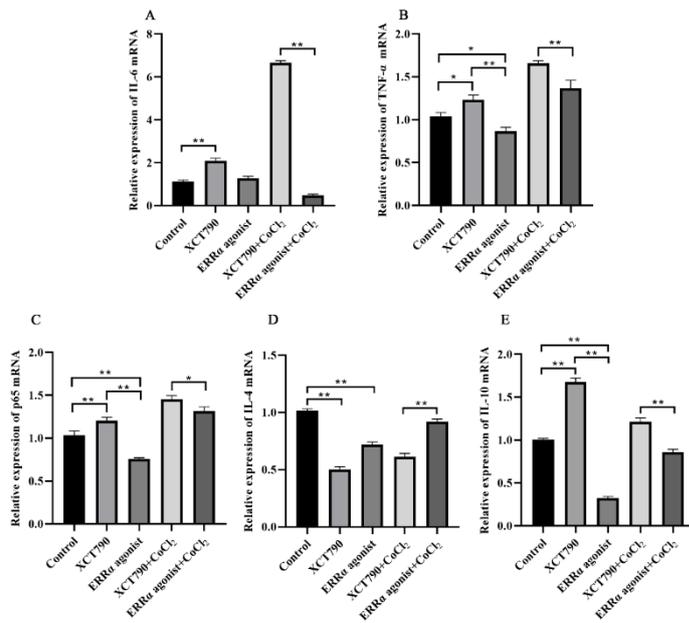
Fig. 7. The mRNA expression in BV2 cells after the corresponding treatments. A– Interleukin 6 (IL-6) mRNA; B – Tumour necrosis factor alpha (TNF-α) mRNA; C – p65 mRNA; D – Interleukin 4 (IL-4) mRNA; E – Interleukin 10 (IL-10) mRNA. Values represent the mean ± standard deviation for three independent experiments. *P < 0.05; **P < 0.01
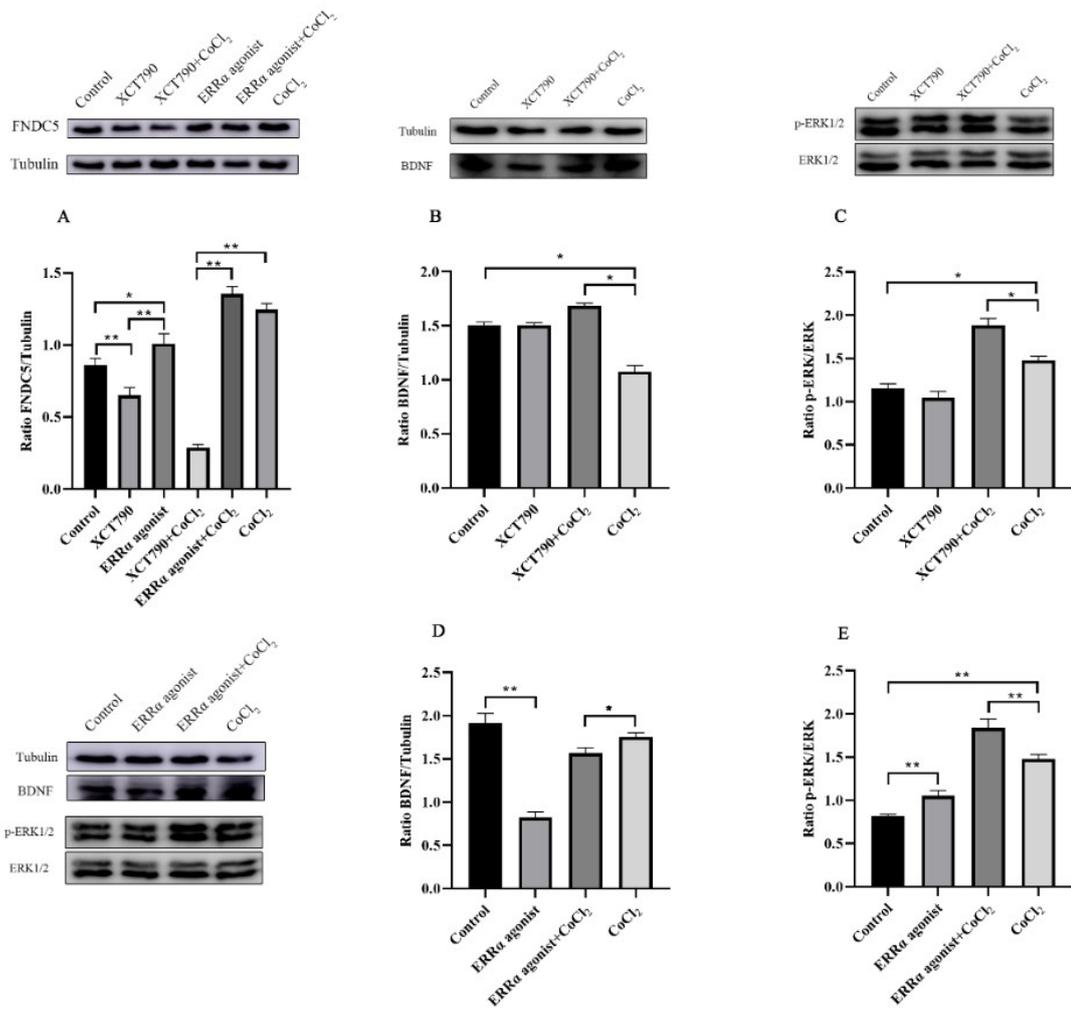

Fig. 8. Expression of fibronectin type III domain containing protein 5 (FNDC5), brain-derived neurotrophic factor (BDNF) and extracellular signal-regulated kinase 1/2 (ERK1/2) in BV2 cells. A – Expression of FNDC5 in the control, XCT790, ERRα) agonist, XCT790+CoCl$_2$, ERRα agonist+CoCl$_2$ and CoCl$_2$ groups; B – Expression of BDNF in the control, XCT790, XCT790+CoCl$_2$ and CoCl$_2$ groups; C – The p-ERK/ERK ratio in the control, XCT790, XCT790+CoCl$_2$ and CoCl$_2$ groups; D – Expression of BDNF in the control, ERRα agonist, ERRα agonist+CoCl$_2$ and CoCl$_2$ groups; E– The p-ERK/ERK ratio in the control, ERRα agonist, ERRα agonist+CoCl$_2$ and CoCl$_2$ groups. Values represent the mean ± standard deviation for three independent experiments. *P<0.05; **P<0.01
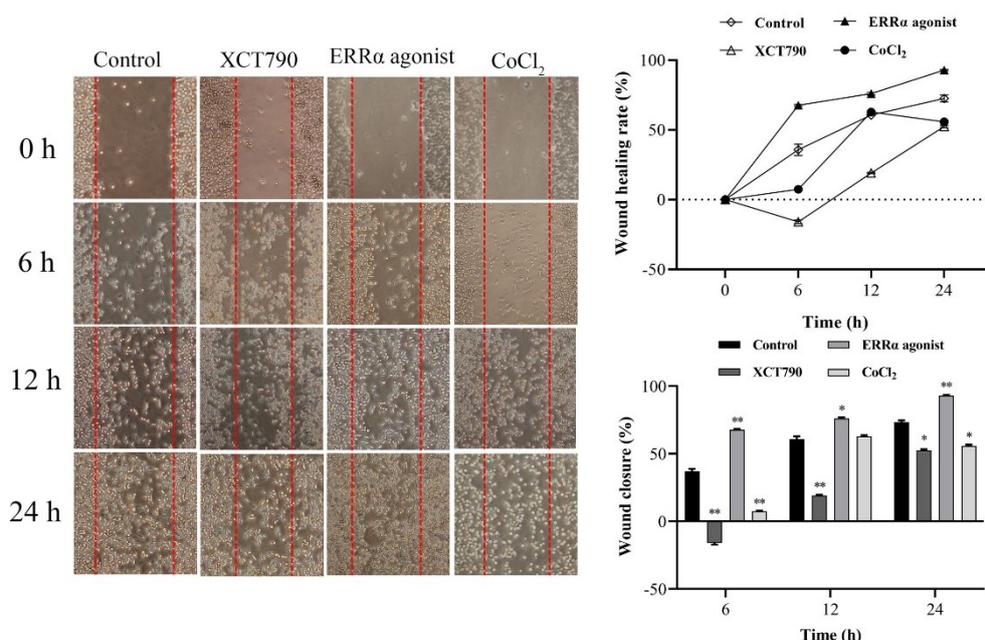
Fig. 9. Wound-healing assay of BV2 cells (400×). Values represent the mean ± standard deviation for three independent experiments. ERRα – oestrogen-related receptor alpha; *P<0.05; **P<0.01

Effect of ERRα on the damage repair and migration of BV2 cells under hypoxic conditions. As shown in Fig. 8, compared with the levels in the control group, the expression levels of FNDC5 and ERK1/2 in the cells treated with CoCl₂ increased significantly (P<0.01), and that of BDNF decreased significantly (P<0.01). The XCT790 group exhibited significantly lower production of FNDC5 (P<0.01) but little different production of ERK1/2 and BDNF compared with the control group, while the ERRα agonist group showed significantly higher production of FNDC5 and ERK1/2 (P<0.01). In relation to secretion of BDNF and ERK1/2 by the cells treated with CoCl₂, this secretion by the cells treated with XCT790+CoCl₂ was significantly potentiated (P<0.01), while secretion of FNDC5 by these cells was significantly attenuated from that of the CoCl₂-treated microglia (P<0.01). The expression levels of BDNF in the cells treated with ERRα agonist+CoCl₂ decreased significantly (P<0.05), and the expression of FNDC5 and ERK1/2 increased significantly (P<0.01). The wound-healing assay revealed that when ERRα was overexpressed, the migration capacity of BV2 cells was enhanced over that of control group cells and their damage repair rate increased (Fig. 9).

Discussion

Spinal cord injury is a traumatic occurrence in the central nervous system, and it is regarded as a major medical challenge owing to the high rate of lasting disability associated with it. After SCI, ischaemia and hypoxia usually occur. Hypoxia is an important factor affecting the prognosis of spinal cord injury (8) as it induces microglial cell activation, which causes a secondary injury. As resident immune cells of the central nervous system, microglia are the first to respond to an SCI. Oestrogen-related receptor alpha, a member of the orphan nuclear receptor transcription factor family, plays a key role in regulating gene expression related to mitochondrial biogenesis, oxidative phosphorylation, glycolysis and fatty acid metabolism (15, 18). When hypoxic injury occurs, cells quickly activate ERRα gene expression to adapt to hypoxic conditions, maintain normal cell functions and activate downstream gene expression (4). Multiple signalling pathways of microglial cells are activated in the hypoxic state and they induce the expression of multiple genes. The most studied inducible factor of oxidative stress injury in cells is HIF-1, which is a heterodimer composed of the oxygen regulatory subunit HIF-1α and the structural subunit HIF-1β. The latter is widely expressed in various mammalian tissues and cells. Under hypoxic conditions, HIF-1α turns on its own molecular switch, fully expresses related genes, regulates cells to adapt to hypoxia and maintains normal cell functions (16). The ERRα–HIF-1α interaction inhibits HIF-1α ubiquitination and reduces HIF-1α degradation (3). In the present findings, the expression of HIF-1α increased after the cells were subjected to a hypoxic treatment (Fig. 4). After the addition of the ERRα inverse agonist XCT790, the expression of HIF-1α decreased (Fig. 4), indicating that ERRα interacts with HIF-1α and ultimately reduces the expression of HIF-1α.

Autophagy is a physiological process in which cells self-degrade, and it can be activated by hypoxia, oxidative stress, nutritional deficiency and other adverse environments. Autophagy protects neurons and causes cell death. Walker *et al.* (24) reported that when autophagy is reduced, recovery is enhanced after SCI.

However, neuronal apoptosis may be inhibited by enhancing autophagy (12). Beclin1, which is central to autophagy regulation, is involved in the formation of autophagosomes (30). LC3 I is hydrolysed and converted to LC3 II during autophagy; therefore, the ratio of LC3 II:LC3 I is a vital marker for autophagy (11). In this experiment, we detected changes in the expression levels of autophagy-related genes that further confirmed the effects of changes in ERRα on the survival rate of hypoxia-damaged microglial cells. Hypoxic damage was found to significantly increase the expression level of the Beclin1 autophagy-related gene and the LC3 II:LC3 I ratio (Fig. 5B), indicating that hypoxia induced autophagy. When the expression of ERRα was inhibited after hypoxic injury, the expression level of the Beclin1 autophagy-related gene and the LC3-II:LC3-I ratio significantly increased (Fig. 5A and B), and when the expression of ERRα was upregulated after hypoxic injury, the same parameters significantly decreased (Fig. 5C and D), indicating that ERRα appropriately induced autophagy after hypoxic injury, thereby playing a protective role in SCI. However, whether there is a two-way regulation of autophagy and inflammatory factor expression remains to be further studied.

Almost all the nucleated cells in the body are affected by changes in oxygen concentration. When the oxygen receptors on the cells are stimulated by hypoxia, they activate various transcription factors and then participate in the regulation of cell signalling pathways. Nuclear factor-kappa B is an important transcription factor that is widely present in a variety of cells. Under normal physiological conditions it has no transcriptional activity. When stimulated by stress such as hypoxia, NF-κB p65 is phosphorylated (20), moves from the cytoplasm to the nucleus, and binds to the corresponding target gene sites in the upper promoter region. This activates its downstream inflammatory factor signalling pathway, which participates in causing hypoxic damage (25). Previous studies have shown that the activation of NF-κB enhances the expression levels of IL-1β and TNF-α, and the increased release of IL-1β and TNF-α in turn activates NF-κB. The activation of NF-κB increases IL-6 synthesis and release, forming a feedback loop that results in increased secondary SCI (22). The hypoxic damage in this experiment significantly increased the phosphorylation levels of p65 in BV2 cells (Fig. 6A), which was similar to the results of an earlier investigation (5). Previous studies have shown that p38MAPK regulates the transcriptional activity of NF-κB (28). In our experiment, the phosphorylation level of p38 increased, and the expression of the IκB-α protein decreased (Fig. 6B and C), suggesting that the activation of the NF-κB signalling pathway in BV2 microglia during hypoxia injury is related to the promotion of IκB-α degradation by p38MAPK. When we changed the expression of ERRα under normal and hypoxic conditions, the results showed that ERRα can inhibit microglia p38 phosphorylation and affect IκB-α

expression and Wu et al. (27) also demonstrated that XCT790 can increase p38 protein expression in triple negative breast tumours. Therefore, we speculate that ERRα might inhibit the activation of the p38 signalling pathway but shows no significant regulatory effect on the p65 protein. In addition, it is pertinent that the expression levels of the proinflammatory cytokines IL-6 and TNF-α significantly decreased (Fig. 7A and B), while the expression level of anti-inflammatory IL-4 increased (Fig. 7D). Given these changes, it may be mooted that ERRα influences the course of secondary inflammatory responses after hypoxic SCI by regulating the expression of anti-inflammatory factors and preventing nerve cells from being overactivated by inflammatory pathways.

FNDC5, a highly glycosylated type I transmembrane protein, is widely expressed, predominantly in muscle cells but also in the central nervous system. Knockdown of FNDC5 in neuronal precursors impaired their development into mature neurons, suggesting a developmental role of FNDC5 in neurons (7). Brain tissue neurotrophy depends, among other factors, on BDNF. During nervous system development, BDNF prevents the damage and death of neurons, improves their pathological state, and plays important roles in promoting their survival and growth. The action of BDNF in regulating the formation of synapses in brain tissue depends on the expression of PGC-1α (14), which is the most important mitochondrial regulator. Wrann et al. (26) showed that PGC-1α regulates neuronal function by affecting BDNF expression through FNDC5, and that ERRα is the upstream regulatory gene of PGC-1α. In the present work, under normal and hypoxic conditions, the expression levels of FNDC5 and BDNF changed in opposite trends (Fig. 8A and B), which was consistent with the results of Wrann et al. (26). Under hypoxic conditions, XC790 inhibits the expression of FNDC5 and promotes the expression of BNDF (Fig. 8A and B), and ERRα agonists have the opposite effect (Fig. 8A and D), suggesting that ERRα can intensify cell proliferation by promoting the expression of FNDC5, which is more conducive to cell repair after hypoxic injury. The inhibition of BDNF expression may be due to the disorder of the PGC-1/FNDC5/BDNF signal pathway caused by hypoxia. The neurotrophic factor cannot complete the negative feedback regulation and compensate to the normal level.

As innate immune cells in the central nervous system, microglia's migration ability is a prerequisite for participating in immune and inflammatory responses. The MAPK family mediates the transmission of extracellular signals to the cell membrane and nucleus, and it regulates almost all processes that stimulate cell proliferation, differentiation, stress responses and apoptosis (29). Extracellular signal-related kinase is a member of the MAPK family that mainly mediates cell proliferation signals (21). Under hypoxic conditions, ERK will be activated and will improve the hypoxia

tolerance of cells, playing a certain protective role (10). The results of this experiment show that under physiological conditions, ERRα can promote ERK1/2 phosphorylation (Fig. 8C) and promote cell damage repair (Fig. 9). This stimulation of phosphorylation by ERRα under normal conditions, means that ERRα affects the transmission of proliferation signals to the nucleus and promotes the proliferation and migration of microglia. However, under hypoxic conditions, this regulatory action was not consistent with the action under normal conditions. It may be that under hypoxic conditions, ERRα may not affect the proliferation of microglia through the ERK 1/2 signalling pathway.

In summary, we contend that ERRα is involved in maintaining the homeostasis of microglia cells by regulating autophagy and the p38MAPK and FNDC5/BDNF signalling pathways when hypoxia occurs.

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