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Effects of PTN on the expression of ERK1/2 and ERK5 in microglia after cerebral ischemia-reperfusion injury

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Abstract: The aim of this study was to investigate the expression of PTN, pERK1/2, PERK5 changes and the relationship between the three. An oxygen-glucose deprivation (OGD) model of microglia was established. At 3h of OGD, cells were collected at 0,15min, 30min,1h, and 2h of reoxygenation, and the expression of pERK5, pERK1/2, pERK1/2, and PTN protein at each time point was detected by western blot. The expression of pERK5 was significantly increased after glucose oxygen stripping and reoxygenation. PERK1/2 expression was also significantly increased after glucose oxygen stripping and reoxygenation compared with the control group. Similar to the trend of changes in PERK5 and PERK1/2, PTN expression increased at 3h after glucose oxygen stripping and 30 min after reoxygenation. The effect of PTN on pERK1/2 protein expression was related to changes in PTN concentration, and the effect of PTN on pERK1/2 protein expression was related to temporal changes, with a 100.21% increase in pERK1/2 expression when PTN (200ng/mL) was administered for 15 min ($p < 0.01$), which peaked. The expression of pERK5 on microglia increases after cerebral ischemia-reperfusion injury. At the appropriate time point, administration of appropriate concentrations of PTN effectively modulated pERK1/2 expression on microglia.
[Chen Jie, Zhang Hao, Wang Bo, Sun Fei, Ma-Qing Fang, Ding-Mao Hua, Tang-Chang Tong, Jiang-Dehua. **Effects of PTN on the expression of ERK1/2 and ERK5 in microglia after cerebral ischemia-reperfusion injury.** *Life Sci J* 2020;17(10):77-81]. ISSN: 1097-8135 (Print) / ISSN: 2372-613X (Online). http://www.lifesciencesite.com. 8. doi:10.7537/marslsj171020.08.

Keywords: PTN; cerebral ischemia-reperfusion injury; microglia

1. Introduction

PTN (Pleiotrophin) is an 18-kd secreted growth factor [1]. It has been demonstrated that PTN is upregulated on microglia in a rat model of cerebral ischemia-reperfusion injury. We then investigated the role of PTN expressed on microglia after cerebral ischemia-reperfusion injury. Recently, several studies have shown that PTN has multiple functions, including regulating cell proliferation and promoting the release of growth factors and neurotrophic factors [2]. Previous studies have shown that PTN induces axonal growth and promotes mitogenesis in fibroblasts, epithelial cells, endothelial cells, and tumor cells. During the differentiation of late retinal progenitor cells, PTN can partially mediate the function of CNTF family cytokines and regulate the stimulatory effect of FGF-2 in human prostate cancer cells. These results strongly suggest that PTN regulates cell proliferation and growth factor production under normal and pathological conditions. Recently, it has been shown that extracellular signal-regulated kinase 1/2 (ERK1/2) is involved in downregulating PTN expression. It is activated and expressed on spinal microglia in neuropathic pain models. However, whether PTN on microglia can activate ERK1/2 and ERK5 remains unreported [3-5]. Little is known about the effects and mechanisms of PTN on microglia after cerebral ischemia-reperfusion. Can PTN activate ERK1/2 and ERK5 on microglia after cerebral ischemia-reperfusion? Can PTN further affect microglia expression and secretion of a range of growth factors, neurotrophic factors and cytokines? If PTN has this effect, by what pathway? It is not known. Therefore in that study, we prepared an in vitro model of microglia ischemia-reperfusion injury to investigate the role of PTN on microglia after cerebral ischemia-reperfusion and the related mechanisms.

2. Material and Methods

Cell culture

Remove the cryotube from the liquid nitrogen or -80°C ultra-low temperature freezer, place it in the 37°C water bath at the fastest speed, and shake the cryotube to completely melt the cell cryotube in the shortest time. After wiping the cryotube with 75% alcohol cotton ball, place it on the super clean bench, transfer the cell cryogenic solution in the tube to a 10ml centrifuge tube, and add 5ml of DMEM solution.

Centrifuge at 1000 r/min for 5 min. The supernatant was discarded, and 5 ml of complete medium was added, gently pipetted evenly into a 100-mm2 cell culture dish, and incubated in a 37°C, 5% CO2 incubator. **Test grouping**

Primary microglia were digested with 0.25% trypsin and passaged to a culture dish with a diameter of 100mm. After the cells were grown to a suitable density, the corresponding treatment factors were given to detect the changes of each indicator at different concentrations and/or at different time points. At 3h after OGD, the effect of reoxygenation on pERK5, ERK5, pERK1/2, Effects of ERK1/2 and PTN protein expression: after OGD, at 0, 15, 30, 60min after reoxygenation to collect cells for protein extraction. Effects of different concentrations of PTN on the expression of pERK5, pERK1/2 and ERK1/2: The cells were randomly divided into 0, 100ng/ml, 200ng/ml, 400ng/ml and 800ng/ml groups. Effects of PTN on the expression of pERK5, ERK5, pERK1/2 and ERK1/2 proteins at different time points: PTN (200ng/ml) was added, and the cells were collected at 0, 15min, 30min, 1h and 2h for protein extraction.

Expression of PTN, ERK1/2, pERK1/2, ERK5 and pERK5 were detected by Western blot

Primary microglia were seeded onto 6-well plates at a density of 1× 10^5/cm2. The proteins were extracted and then subjected to gel electrophoresis, and the nitrocellulose membrane with the target protein was removed and washed three times with TBST for 5-10min. The membrane was then immersed in TBST blocking solution containing 5% nonfat dry milk at room temperature on a shaker 1-2h. After decanting the blocking solution, cut off the band corresponding to the molecular weight of target protein according to the molecular weight labeled by Mark, and respectively place them in the primary antibody diluted by new TBST blocking solution: (1) pERK1/2 (1:2000); (2) ERK1/2 (1:1000); (3) PERK5(1:4000); (4) ERK5(1:1000); (5) PTN (1:200); (6)β-actin (1:2000). The membranes were blocked for 1h at room temperature on a shaker and then incubated overnight in a refrigerator at 4°C. The next day, the primary antibody blocking solution was carefully decanted and washed four times with TBST for 5-10min. Membranes were placed in blocking solution containing 5% nonfat dry milk in TBST and incubated for 1h at 25°C with anti-rabbit (1:1000) and anti-mouse (1:1000) secondary antibodies conjugated to horseradish peroxide. Membranes were washed six times with TBS for 10 min. The membrane was exposed to ECL fluorescent kit.

Statistical analysis

All experiments were repeated at least three times, using SPSS25.0 Statistical analysis software performed statistics on all data of Western blot results. Differences between the groups were analyzed by one-way analysis of variance (ANOVA), and pairwise comparisons were analyzed by t-test. Statistical significance was considered at $P < 0.05$, and all data were expressed as mean ± standard deviation (x ± SD).

3. Results

The effects of glucose and oxygen stripping and reoxygenation on the expression of pERK5 and ERK5 on microglia.

The OGD model of microglia was established by treating primary microglia according to the methods of glucose oxygen stripping and reoxygenation described in Materials and Methods. Inlet a mixture of 5% CO2,10% H2,85% N2 into the box After 3h, take out the culture plate, add complete culture medium, place it in 37°C CO2 incubator (5% CO2, 95% air) for continuous culture, respectively collect the cells at 0, 15min, 30min, 1h, 2h, and detect the protein expression of pERK5 and ERK5 at each time point. Figure 1.

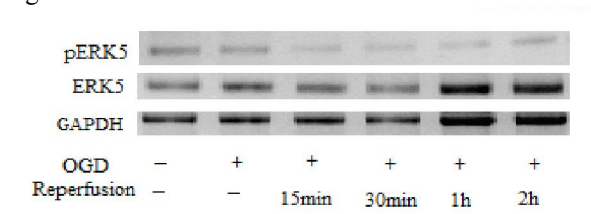

Figure 1. Effect of OGD and reperfusion on pERK5 and ERK5 expression in microglia.

We found OGD reperfusion significantly induced pERK5 expression at 30 min (273.71±14.88%, $p<0.01$) and 1 h (125.56±18.79%, $p<0.05$).

The effects of glucose and oxygen stripping and reoxygenation on the expression of pERK1/2 and ERK1/2 on microglia.

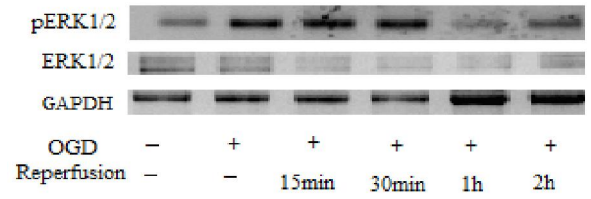

Figure 2. Effect of OGD and reperfusion on pERK1/2 and ERK1/2 expression in microglia.

The OGD model of microglia was established by treating primary microglia according to the methods of glucose oxygen stripping and reoxygenation described in Materials and Methods. Inlet a mixture of 5% CO2, 10% H2, 85% N2 into the box At 0, 15min, 30 min, 1h and 2h, the cells were cultured in a 37° C CO2 incubator (5% CO2, 95% air) and the expression of

pERK1/2 and ERK1/2 protein was detected. Figure 2.

The pERK1/2 expression was increased by 38.66% at 15 min ($p<0.05$) and by 342.38% at 30 min ($p<0.01$).

The effects of glucose and oxygen stripping and reoxygenation on PTN expression on microglia.

The OGD model of primary microglia was established according to the methods of glucose oxygen stripping and reoxygenation described in Materials and Methods. Inlet a mixture of 5% CO2, 10% H2, 85% N2 into the box After 3h, the culture plate was removed, complete culture medium was added, and the culture was continued in a 37 ° CCO2 incubator (5% CO2, 95% air), and the cells were collected at 0, 15min, 30min, 1h, and 2h, respectively, and the PTN protein expression was detected at each time point. See Figure 3.

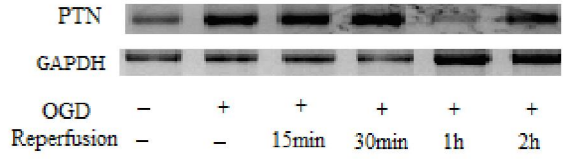

Figure 3. Effect of OGD and reperfusion on PTN expression in microglia. Similarly, PTN protein level was increased by 335.68% at 30 min ($p<0.01$) and by 54.50% at 60 min ($p<0.05$).

Effect of PTN on the expression of PERK5, PERK1/2.

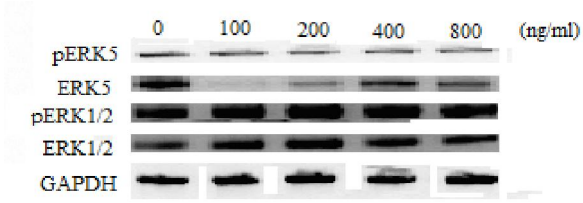

Figure 4. The effect of various concentrations of PTN on pERK5 and pERK1/2 expression.

From the above results, we can see that the change trend of pERK5, pERK1/2 and PTN is similar after oxygen stripping for 3 hours and reoxygenation for glucose. Therefore, we wondered whether PTN is involved in the regulation of PERK5 and PERK1/2 expression. Therefore, we determined the effect of PTN at different concentration gradients and different time gradients on the expression of PERK5 and PERK1/2 proteins. We first selected different concentrations of PTN (0,100ng/ml, 200ng/ml, 400ng/ml, 800 ng/ml) on microglia 15min (Figure 4). Based on the results, we selected the optimal PTN concentration (200 ng/mL) and applied it to microglia for 0, 15min, 30min, and 60min. To measure the effect of different time gradients on PERK5 and PERK1/2 expression (Figure 5).

The pERK1/2 protein levels was concentration-dependent and reached maximum following exposure to 200 ng/ml PTN (172.26% elevation of pERK1/2, $p<0.01$). But pERK5 varied little among each PTN concentration ($p>0.05$).

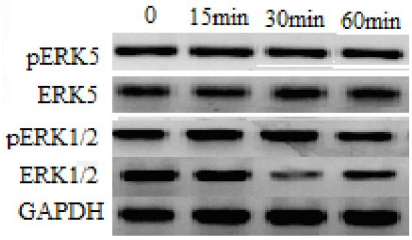

Figure 5. The effect of different time points of PTN (200 ng/ml) on pERK5 and pERK1/2 expression.

PTN significantly promoted the pERK1/2 protein expression in a time-dependent manner, with the maximum effect (100.21% increase, $p<0.01$) observed at 15min, relative to untreated control. But pERK5 varied little at each time point ($p>0.05$).

4. Discussions

Studies have found increased PTN expression on microglia after cerebral ischemia-reperfusion in rats [6]. It has recently been shown that in osteoblasts, ERK1/2 is involved in downregulating PTN expression. ERK5 is activated and expressed on spinal microglia in neuropathic pain models. However, after cerebral ischemia-reperfusion, whether ERK5 expression changes on microglia and whether PTN can activate ERK1/2 and ERK5 and whether PTN can directly activate microglia have not been studied [7]. PTN expression is tightly regulated and only transiently expressed at the appropriate time and place, and this expression is cell specific. PTN MRNA gradually increases during embryogenesis, peaks after birth, and remains at this high level until adulthood. At the mature body stage, PTN expression is restricted to the central nervous system [8 -9]. PTN is highly conserved and plays an important role in regulating neural tissue development and repair [10]. Our study found that PTN expression was also significantly increased after glucose oxygen stripping and reoxygenation, suggesting that PTN may be involved in tissue repair after cerebral ischemia-reperfusion injury.

PTN signaling can act through three cell surface receptors, syndecan-3, anaplastic lymphoma enzyme (ALK), and protein tyrosine phosphatase receptor (RPTPβ/ζ). It is widely expressed on almost all human breast cancer cell lines and plays an important role in tumor cell metastasis and adhesion [11]. PTN can fulfill multiple functions through RPTPβ/ζ in regulating cell

growth, proliferation, migration, and mesenchymal-epithelial transition [12]. It has been shown that on endothelial cells, PTN can activate ERK1/2 expression through RPTPβ/ζ[13].

Our study is similar to the above findings in that PTN was found to activate ERK1/2 on microglia. This may also be achieved through the RPTPβ/ζ pathway. It was found that the effect of PTN on the expression of pERK1/2 protein was related to the change of PTN concentration and time gradient. When microglia were treated with PTN at a concentration of 200 ng/ml for 15min, the expression of pERK5 and pERK1/2 protein reached the peak. It has been confirmed that ERK1/2, as a classical member of MAPK family, is involved in the regulation of a range of cellular functions, such as cell metabolism, activity, and cell death. ERK1/2 can be involved in the regulation of cell proliferation and differentiation, and plays an important role in the regulation of cellular responses caused by growth factor stimuli such as NGF and PDGF [14]. Mitogen-activated protein kinases (MAPKs), which can be involved in regulating various physiological functions of cells by transmitting extracellular signals into the nucleus. A total of four subclasses of the MAPK family in mammals have been identified, including: ERK, p38, c-Jun amino-terminal kinase (JNK), and ERK5/BMK1 (big MAP kinase 1) [14]. After cells are stimulated by extracellular stimuli, upstream kinases activate different MAPK subfamilies and participate in the regulation of stress, inflammation, cell growth, division, differentiation, death and functional synchronization between cells by phosphorylating various substrates such as transcription factors, cytoskeleton-related proteins and enzymes. In the process of regulation, the MAPK signal transcription system has two distinctive features: one is the activation through the phosphorylation of threonine and tyrosine in VIII region. The second is a Ser/Thr protein kinase mediated by proline and has a Ser-Thr-Pro minimal common target sequence [15]. The MAPK signal transduction process is carried out through a highly conserved three-tiered kinase cascade, which includes three key kinases mitogen activated protein kinase kinase kinase (MAPKKK), MAPKK and MAPK. MAPKKK is activated upon phosphorylation in response to mitogen stimulation. Subsequently, MAPKKK, in turn, activates MAPKK through phosphorylation, and finally MAPK is activated by phosphorylation of MAPKK and translocated into the nucleus [16]. MAPK in the nucleus regulates the activity of many substrates by phosphorylating their serine/threonine residues. PERK1/2 levels are usually elevated after cerebral ischemia-reperfusion injury. Its cytoprotective effect is mainly achieved by participating in the processes of growth factors, estrogen, and preconditioning on ischemic brain [17]. Our study showed for the first time that pERK5 expression on microglia was significantly increased by glucose oxygen stripping and reoxygenation. ERK5, also known as BMK1, has twice the molecular weight of other MAPK family members, and both ERK5 and ERK1/2 are MAPK family members with similar structures. ERK5 can be widely expressed in various tissues, and Wangrm et al found that ERK5 expression was increased in the hippocampus after transient global ischemia in rats, and was mainly concentrated in the CA3/DG region rather than in CA1 pyramidal cells. And ERK5 plays a protective role in neuronal cells damaged in the CA3/DG region induced by ischemia [18-20]. Our study also found that different concentrations of PTN and different time gradients had little effect on PERK5 expression, indicating that PTN could not activate ERK5. Whether ERK5 can be activated by additional upstream signal transduction proteins and its exact molecular mechanism need to be further explored. In addition, pERK1/2 and PTN expression were also significantly increased after glucose oxygen stripping and reoxygenation. The changes of PTN, PERK5 and PERK1/2 were similar. The expression of PTN, PERK5 and PERK1/2 reached the peak at 3 hours after glucose and oxygen stripping and 30min after reoxygenation. ERK1/2, which depends on its rapid activation, started to be expressed at 15min after reperfusion, reached a peak at 30min, and gradually returned to normal levels after 1h. However, PTN and ERK5, which peaked at 30min after reperfusion, gradually partially recovered after 1h and then returned to normal levels after 2h. These results suggest that the effects of ERK1/2 are rapid and transient, whereas PTN and ERK5 are relatively slow and persistent.

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10/19/2020