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The Effect of Erythropoietin on Neurotrophic Factors in N9 Murine Microglial Cells*

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Aim: In this study, we investigated whether interferon gamma (IFNγ), lipopolysaccharides (LPS) and amyloid beta (AMYβ), as toxic stimulator agents, and erythropoietin (EPO), as a neurotrophic agent, have an effect on the production of the following neurotrophic factors in the N9 murine microglia cell line: neurotrophin 3 (NT3), neurotrophin 4 (NT4), and brain-derived neurotrophic factor (BDNF).

Materials and Methods: Microglial cells were incubated with 50 µg/ml AMYβ, or 1 µg/ml of LPS plus 100 U/ml recombinant murine IFNγ, and/or one of three different concentrations (0.1, 1.0, and 5.0 U/ml) of recombinant mouse EPO for 24 h.

Results: EPO 0.1 U/ml dose significantly increased NT4 levels compared to EPO 5.0 U/ml dose ($P < 0.05$). EPO, in all doses, and AMYβ significantly induced NT4 secretion in microglias, while BDNF and NT3 were not changed by AMYβ or EPO. LPS + IFNγ alone did not change neurotrophic factor levels in any group. However, EPO with LPS and IFNγ induced NT4 secretion, especially the 5.0 U/ml dose of EPO.

Conclusions: NT4 secretion, which was markedly induced by exposure to both AMYβ and EPO in N9 murine microglias, may be an important result for neuronal survival. These results suggest that inflammatory mechanisms in microglia may also involve the neuroprotective response of these cells; this may be a promising area of study of neurodegenerative processes.

Key Words: Microglia, N9 cell line, murine, erythropoietin, neurotrophic factors

N9 Fare Mikroglial Hücrelerinde Nörotrofik Faktörler Üzerine Eritropoetinin Etkisi

Amaç: Bu çalışmada N9 murin mikroglia hücre hattında toksik uyarıcı ajanlar olarak interferon gama (IFNγ), lipopolisakkarid (LPS), amiloid beta (AMYβ) ve nörotrofik ajan olarak eritropoetin (EPO)'in nörotrofik faktörler olan nörotrofin 3 (NT3), nörotrofin 4 (NT4) ve beyin kökenli nörotrofik faktör (BDNF) üretimi üzerine etkisi olup olmadığını araştırdık.

Yöntem ve Gereç: Mikroglial hücreler 50 µg/ml AMYβ veya 1 µg/ml of LPS + 100 U/ml IFNγ ve/veya EPO'nun üç ayrı konsantrasyonu (0.1, 1.0 ve 5.0 U/ml) ile 24 saat inkübe edildi.

Bulgular: EPO'nun 0.1 U/ml dozu, EPO 5.0 U/ml dozuna göre, NT4 düzeylerini daha anlamlı olarak arttırmıştır ($P < 0.05$) EPO'nun her üç dozu ve AMYβ mikroglial NT4 sekresyonunu anlamlı olarak indüklerken; BDNF ve NT3 düzeyleri AMYβ veya EPO ile değişmedi. LPS + IFNγ uygulaması tek başına herhangibir grupta nörotrofik faktör düzeylerini değiştirmedi. Fakat, EPO ile birlikte LPS + IFNγ uygulaması, NT4 salınımını, özellikle EPO'nun 5.0 U/ml dozunda arttırdı.

Sonuç: N9 murin mikroglia hücre hattında AMYβ veya EPO uygulanımının NT4 salınımını indüklemesi, nöronal hayatta kalım için önemli bir sonuç olabilir. Bu bulgular, mikrogliada inflamatuar mekanizmaların bu hücrelerin nöroprotektif cevabını da kapsayan; belki de nörodejeneratif olaylarla ilgili çalışmalara da ışık tutacak bir alan olabileceği yönündedir.

Anahtar Sözcükler: Mikroglia, N9 hücre hattı, murin, eritropoetin, nörotrofik faktörler

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Introduction

A glia-mediated, inflammatory immune response is an important component of the neuropathophysiology of neurodegenerative diseases such as Alzheimer's disease (AD) and Parkinson's disease (PD). Activated microglia can mediate neuronal cell death and the repair processes of central nervous system (CNS) injuries. Recent studies suggest that the activation of microglia in response to injury, illness, aging, or other causes begins a cascade of events that can best be characterized as an inflammatory process. This cascade is mediated at first by the proinflammatory cytokine interleukin 1, which is overexpressed by the activated microglia. Microglias produce and release certain growth factors that are essential for neuron survival, such as erythropoietin (EPO) and interferon gamma (IFNγ) (1,2). Amyloid deposition, inflammation and regenerative mechanisms are also early pathogenic events in experimental models (3,4).

Erythropoietin (EPO) was first characterized as a hematopoietic growth factor and has been in clinical use for over a decade for the treatment of anemia. EPO is currently named "NeoRecormon", and since its receptor was found in glial cells, it was assumed to have beneficial effects on neuronal cells (5-7). The neuroprotective effects of EPO have been demonstrated *in vitro* and *in vivo* in a variety of CNS injury models, such as focal cerebral ischemia and chemical–induced neurotoxicity (8-15). In CNS cell culture studies, EPO was shown to be involved in the regulation of intracellular calcium and signal mechanisms; the enhancement of calcium-dependent or nondependent neurotransmitter release may explain its neuroprotective efficacy. EPO has also been proposed to be an antioxidant, anti-inflammatory and trophic agent in the CNS due to its stimulation of neuronal supportive cells and microglias and it increases their viability (12-14,16). Another possibility is that EPO combined with another cytokine such as IFNγ and lipopolysaccharides (LPS) or various neuroactive drugs stimulate glial cells maintaining support for neurons by affecting neurotrophic factors and nitric oxide (NO) production (8,9,15). In our previous studies, we demonstrated that EPO increased antioxidant enzyme activity and NO production in the substantia nigra in a model of methyl-4-phenyl-1,2,3,6–tetrahydropyridine (MPTP)-induced parkinsonism in C57BL mice (10,11). Neurotrophic factors, such as nerve growth factor (NGF), neurotrophin 3 (NT3), neurotrophin 4 (NT4), and brain-derived neurotrophic factor (BDNF), in addition to their neurotrophic actions, may also regulate microglial dynamics, thereby influencing the surrounding milieu during neuronal inflammation and regeneration. In this study, we investigated whether microglial activation with IFNγ + LPS or amyloid beta (AMYβ) as toxic stimulator agents, and with EPO as a neurotrophic agent, has an effect on the production of neurotrophic factors (BDNF, NT3 and NT4) in an N9 murine microglial cell line.

Materials and Methods

Microglial Cultures

The N9 murine microglia cell line was kindly provided by Paola Ricciardi-Castagnoli (Cellular Pharmacology Center, Milan, Italy) and maintained with Roswell Park Memorial Institute (RPMI) medium supplemented with 5% fetal calf serum, 100 U/ml penicillin and 100 mg/ml streptomycin. The purity of the microglia cultures was assessed by immunostaining with monoclonal antibody against CD11b/c (Invitrogen, Caltag Laboratories, California, USA), and more than 90% of the cells were positively stained.

Microglial cells were incubated with 50 mg/ml AMYβ (Sigma-Aldrich, Taufkirchen, Germany), or 1 mg/ml LPS (from *Escherichia coli*; Sigma-Aldrich, Taufkirchen, Germany) plus 100 U/ml recombinant murine IFNγ (Roche Diagnostics, Basel, Switzerland) and/or one of three different concentrations (0.1, 1.0, and 5.0 U/ml) of recombinant mouse EPO (Roche Diagnostics, Basel, Switzerland) for 24 hours (h). None of the cytokines was added to the control culture. In summary, the nine treatment groups (three cell lines in each group) were as follows:

1) Control
2) LPS (1 mg/ml) + IFNγ (100 U/ml)
3) AMYβ (50 mg/ml)
4) EPO (0.1 U/ml)
5) EPO (1.0 U/ml)
6) EPO (5.0 U/ml)
7) LPS (1 mg/ml) + IFNγ (100 U/ml) + EPO (0.1 U/ml)
8) LPS (1 mg/ml) + IFNγ (100 U/ml) + EPO (1.0 U/ml)
9) LPS (1 mg/ml) + IFNγ (100 U/ml) + EPO (5.0 U/ml)

Neurotrophic Factor Determinations

After incubation for 24 h, BDNF, NT3 and NT4 levels were duplicately measured by ELISA methods (Promega Inc, Madison, WI, USA). Microplates were coated with polyclonal antibodies specific to the neurotrophic factor to be analyzed, which were diluted in carbonate coating buffer (0.025 M sodium bicarbonate, 0.025 M sodium carbonate, pH = 9.7) by incubating for 18 h at 4°C. Nonspecific binding was prevented by adding a blocking solution containing 1M phosphoric acid. The captured antigens were bound by a second specific monoclonal antibody (mAb). After washing, the amount of specifically bound mAb was then detected using anti-rat IgG conjugated to horse radish peroxidase as a tertiary reactant. The unbound conjugate was removed and following incubation with chromogenic substrate 3,3,5,5-tetramethylbenzidine, the color change at 450 nm was recorded, as this indicated the amount of neurotrophic factors in each well.

Statistical Analysis

Comparisons were made using Mann-Whitney U test and one-way analysis of variance (ANOVA), and Spearman correlation coefficient by SPSS 10.0® (SPSS, Inc., Chicago, IL, USA). P values <0.05 were considered statistically significant.

Results

BDNF: AMYβ did not significantly change BDNF levels compared to controls (P = 0.075). Similarly, EPO, LPS + IFNc and LPS + IFNγ + EPO did not change BDNF levels a significant amount (P > 0.05) (Table 1). A significant positive correlation was found between BDNF and NT3 (P < 0.0001; r = 0.667).

NT3: AMYβ did not significantly change NT3 levels compared to the controls (P = 0.067). EPO, LPS + IFNC, and LPS + IFNC + EPO did not change NT3 levels significantly (P > 0.05) (Table 1).

NT4: The lowest NT4 levels were found in the control group, while the highest levels were observed in the EPO 0.1 U/ml group. EPO at 0.1, 1.0 and 5.0 U/ml doses increased NT4 levels significantly compared to controls (P < 0.000 for all doses) and the levels of NT4 of the EPO 0.1 group were significantly higher than those of EPO 5.0 U/ml group (P < 0.05). AMYβ significantly increased NT4 levels compared to the control group (P < 0.05 or less for each group). Although levels of NT4 after LPS + IFNγ treatment were not significantly different than that of the controls, microglial activation by LPS + IFNγ + EPO (0.1, 1.0 and 5.0 U/ml) significantly increased NT4 levels compared to controls (P < 0.05, P < 0.05 and P < 0.0001, respectively). Of these groups, LPS + IFNγ +

Table 1. Levels of microglial brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT3) and neurotrophin-4 (NT4) levels (pg/ml) after exposure to amyloid beta (AMYβ), lipopolysaccharides (LPS) + interferon gamma (IFNγ), and erythropoietin (EPO). BDNF and NT3 results are reported as mean ± SD while NT4 results are median.

Groups	BDNF	NT3	NT4
Control	3933 ± 388	1000 ± 98	501[c]
LPS + IFNγ	3583 ± 782	725 ± 240	504
AMYβ	4433 ± 759	1170 ± 166	1164d
EPO (0.1 U/ml)	3167 ± 580	952 ± 65	1859[a,b]
EPO (1.0 U/ml)	2817 ± 1327	1034 ± 192	1656[a]
EPO (5.0 U/ml)	3583 ± 586	996 ± 51	1439[a]
LPS + IFNγ + EPO (0.1 U/ml)	2817 ± 617	803 ± 82	1400[d]
LPS + IFNγ + EPO (1.0 U/ml)	3383 ± 858	956 ± 172	1223[d]
LPS + IFNγ + EPO (5.0 U/ml)	3167 ± 231	1394 ± 537	1658[a,e]

[a] P < 0.0001, significantly higher than the control and LPS + IFN groups.
[b] P < 0.05, significantly higher than the EPO 5.0 group.
[c] P < 0.05, significantly lower than the other groups except LPS + IFNγ group.
[d] P < 0.05, significantly higher than the control and LPS + IFNγ groups.
[e] P < 0.01 significantly higher than the EPO 5.0 group.

EPO 5.0 U/ml had a greater effect than the other only EPO groups, and NT4 levels in the LPS + IFNγ + EPO 5.0 U/ml group were significantly higher than those of the only EPO 5.0 U/ml group ($P < 0.01$) (Table 1).

Discussion

Neurotrophic factor expressions were investigated in the pathogenesis of neurodegenerative diseases and were found useful for both neuroactive drug screening *in vitro* and possibly cell therapy for neurodegenerative diseases (17,18). Cortical BDNF levels were upregulated, showing an over 10-fold increase compared with age-matched controls in AD. BDNF upregulation was significantly correlated with the AMYβ load, and thus the authors implicated this NT as playing an important role in the regulation of neuronal survival, axonal regeneration processes and neurological disorders, including epilepsy (19-24). In PD, glia may upregulate NTs like BDNF, NT3, NT4 and the tyrosine kinase receptors, TRKB and TRKC, in response to signals released mainly from activated microglial cells, failing nigral neurons and, to a lesser extent, from reactive astrocytes (25). This glial response may be the source of neurotrophic factors and can protect against reactive oxygen species including NO and glutamate (26). Alternatively, this glial response can also cause release of deleterious pro-oxidant reactive species and pro-inflammatory prostaglandins, LPS and IFNγ (25). These are also powerful inducers of NGF and BDNF production in mouse astrocyte cultures and thus play a neuroprotective role in the CNS (27). Likewise, in brain microglial cells, LPS modulate the expression of both NT3 and TRKC, which play pivotal roles in inflammatory and degenerative disorders (15). Some studies indicated that LPS induce microglial NGF expression and also analyzed the effects of the inflammatory agent on TRKA, the high-affinity receptor for NGF (28,29). In particular, it was reported that microglial NGF induces death of retinal ganglion cells (30). Therefore, it remains to be determined whether an induction in microglial NT expression during inflammation is beneficial or detrimental for neuronal or non-neuronal cells. Miwa et al. (31) demonstrated that microglias express mRNAs of BDNF and NT4 but not NT3 by LPS induction. Their results for NT3 and NT4 are inconsistent with those of Elkabes et al. (32), who showed that LPS induced microglial NT3 expression, while BDNF and NT4 expression were unaltered by LPS.

Proinflammatory cytokines were found to regulate expression of EPO and EPO receptor in human neurons, astrocytes, and microglia and further facilitate interactions among different cell types in the human CNS (18). Treatment with EPO inhibited the expression of iNOS mRNA and nitrite production resulting from proinflammatory stimulation by IFNγ and LPS in rat oligodendrocytes (8). Park et al. (9) revealed that EPO promoted neuronal cell differentiation through increased release of NGF from astrocytes, and this effect may be associated with signals of an extracellular signal-regulated kinase pathway. Neuronal differentiation was even further promoted when the neuronal stem cells were cultured with an astrocyte culture medium treated with a 10 U/ml dose of EPO. Compared to untreated astrocytes, EPO-treated astrocytes increased about two-fold in beta-NGF, but did not raise BNDF and NT3 levels. Similar to the study of Park et al., we also found in our study that BDNF and NT3 levels were unchanged and positively correlated with each other in microglias.

Wang et al. (33) showed that EPO treatment significantly increased brain levels of BDNF, improved functional recovery, and increased the density of cerebral microvessels at the site of stroke. Zhang et al. (34) found that EPO improved functional recovery after experimental allergic encephalomyelitis in mice, possibly via stimulating oligodendroglial BDNF expression. EPO also affects the ability of oligodendrocytes to promote myelin repair in the normal and damaged adult CNS.

It was reported by Viviani et al. (35) that exposure of primary hippocampal neurons to EPO significantly increased BDNF after 1 h. BDNF mRNA levels further increased up to 4 h, to return to control levels after 18 h of EPO treatment. Miwa et al. (15) found an increased BDNF and NT4 mRNA expression in primary microglia after LPS treatment for 12 h. Olivieri et al. (36) reported increased BDNF and NT4 levels in SHSY5Y cell lines after 12-h AMYβ treatment. Mizuno et al. (37) found decreased BDNF mRNA levels after 24 h incubation of primary microglias with nicergoline. Elkabes et al. (32) reported an increase in NT3 and NGF with LPS continuing up to 24 h, while no change was noted in BDNF and NT4 levels. For the incubation period (24 h) selected in our study, the studies of Mizuno (37) and Elkabes (32) were taken as reference.

Both "EPO alone" groups and EPO + LPS + IFNγ group activated microglia and significantly increased NT4

levels compared to the control. However, the latter's significance was less than that of the former. We also found that increases in NT4 were more evident at the 0.1 U/ml and 1.0 U/ml doses of EPO, which is similar to the findings of Wen et al. (38). The enhanced NT4 secretion in microglial cells after exposure to AMYβ may be an inflammatory reaction against this toxic agent. That is, microglias produce an immunological response to inflammation caused by AMYβ, which is a molecule responsible for neuronal injury in neurodegeneration. EPO is more neuroprotective at lower doses and may also be a useful agent for exploring the regulatory mechanism of NT4 synthesis in murine microglias.

AMYβ is a toxic and EPO is a protective agent; both of them significantly increased NT4 levels compared to the control. Ending with similar results can be speculated to arise by the following mechanisms: as a defense reaction against AMYβ, microglial cells could have secreted neurotrophic factor; on the other hand, neurotrophic factor release could be the result of one of EPO's protective effects. Chong et al. (39) showed that EPO treatment protected the primary hippocampal neurons from apoptotic tissue injury in a rat model of AMYβ toxicity. In their study, EPO in a concentration range of 0.001 ng/ml to 1000 ng/ml was not toxic to neurons. 1-50 ng/ml concentrations of EPO enhanced neuronal survival. However, EPO concentration <1 ng/ml or >50 ng/ml did have any positive effect on neuronal survival during AMYβ administration. Chong et al. (39) showed that in a concentration-specific manner, EPO was able to protect DNA integrity and cellular membrane asymmetry during AMYβ exposure. Additional studies in a number of cell models also illustrate a tight therapeutic concentration range for EPO (14,40-42). In the present study, AMYβ significantly increased NT4 levels compared to controls. Although it also caused increases in BDNF and NT3, these were not significant. These results may indicate a reactive inflammatory response against damage induced by AMYβ, and having significant changes only in NT4 could be the consequence of differing receptor sensitivities and receptor densities of different glial cell types.

In summary, our study results reveal that both AMYβ and EPO stimulate murine microglial cells, maintaining support for neurons by increasing NT4 production, which is known to facilitate neuronal survival. Targeting microglias as mediators for neuroprotective drugs may be a promising strategy in the treatment of neurodegenerative processes.

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