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Protective action of erythropoietin on neuronal damage induced by activated microglia

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Inflammation is a physiological defense response, but may also represent a potential pathological process in neurological diseases. In this regard, microglia have a crucial role in either progression or amelioration of degenerative neuronal damage. Because of the role of hypoxia in pro-inflammatory mechanisms in the nervous system, and the potential anti-inflammatory protective effect of erythropoietin (Epo), we focused our investigation on the role of this factor on activation of microglia and neuroprotection. Activation of microglial cells (EOC-2) was achieved by chemical hypoxia induced by cobalt chloride ($CoCl_2$) and characterized by increased levels of nitrite, tumor necrosis factor-α and reactive oxygen species production, as well as up-regulation of inducible nitric oxide synthase expression. Under these conditions, cell proliferation data and proliferating cell nuclear antigen (PCNA) staining demonstrated a mitogenic effect of chemical hypoxia. Even though pre-treatment with Epo did not prevent nitrite production, inducible nitric oxide synthase protein expression or tumor necrosis factor-α secretion, it prevented the oxidative stress induced by $CoCl_2$ as well as cell proliferation. Neuronal cells (SH-SY5Y) cultured in the presence of conditioned medium from activated EOC-2 cells or macrophages (RAW 264.7) developed significant apoptosis, an effect that was abolished by Epo via Epo/Epo receptor activation. The results show that even though Epo did not exert a direct anti-inflammatory effect on microglia activation, it did increase the resistance of neurons to subsequent damage from pro-inflammatory agents. In addition to its anti-apoptotic ability, the Epo antioxidant effect may have an indirect influence on neuronal survival by modulation of the pro-inflammatory environment.

Introduction

Inflammation is a physiological defense response against microorganisms or injury. However, it represents a potential pathological process in several neurological diseases [1–5]. In most neurodegenerative disorders, including Parkinson's and Alzheimer's diseases, multiple sclerosis, and AIDS-related dementia, massive neuronal cell death occurs as a consequence of an uncontrolled inflammatory response, in which activated microglia and their cytotoxic agents appear to play a crucial role.

In response to injury in the central nervous system, microglia activation represents a potential pathological

Abbreviations
CM, conditioned medium; DCFH-DA, 2′,′-dichlorofluorescin diacetate; Epo, erythropoietin; EpoR, erythropoietin receptor; IFNγ, interferon-γ; iNOS, inducible nitric oxide synthase; LPS, lipopolysaccharide; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NO, nitric oxide; PI3K, phosphatidylinositol 3-kinase; ROS, reactive oxygen species; TNF-α, tumor necrosis factor-α.

process, releasing inflammatory mediators such as nitric oxide (NO), prostaglandins, cytokines and reactive oxygen species (ROS) [3–5]. In brain damage, such as cerebral ischemia, hypoxia causes neuronal cell injury by both direct action and an indirect effect of microglia activation [6]. Thus, it is likely that neuronal death may be exacerbated by toxic inflammatory mediators produced by activation of microglia [7,8].

Because current treatments for the above-mentioned neurodegenerative diseases are not effective, a number of regulatory molecules acting as microglia-deactivating factors have been the focus of considerable research [9]. Erythropoietin (Epo) is well known as the growth factor that maintains the number of circulating erythrocytes, primarily by preventing apoptosis of erythroid progenitors. However, its biological role has been expanded by the finding of specific receptors in non-hematopoietic tissues. In the nervous system, Epo receptors have been found in various cell types, such as neurons, microglia, astrocytes and endothelial cells [10,11]. *In vitro* studies have shown neuroprotective effects of Epo against apoptosis induced by various agents, such as glutamate, hypoxia, tumor necrosis factor-α (TNF-α) or glucose/serum deprivation [12–14]. These results were supported by studies in *in vivo* models [15,16].

Previously, we have described the protective action of Epo on immature SH-SY5Y neuroblastoma cells against direct damage induced by staurosporine, TNF-α or hypoxia. Our results suggest that Epo may repress neuronal apoptosis by activation of phosphatidylinositol 3-kinase (PI3K) and nuclear factor κB signaling pathways, and up-regulation of the expression of anti-apoptotic factors such as Bcl-2 and Bcl-xL [14,17,18].

With regard to microglial tissue, inconclusive data are available. Epo provided direct protection of the EOC-2 microglial cell line against early and late apoptotic signs, such as membrane phosphatidylserine exposure and genomic DNA degradation [19]. It has also been suggested that Epo may exert an anti-inflammatory action by reducing monocytic infiltration and microglia activation [10,20]. However, recent evidence showed that Epo was unable to prevent the production of inflammatory mediators such as NO and inflammatory cytokines in rat microglial cells and the murine microglia cell line BV-2 [21].

It is recognized that hypoxia augments inflammatory cytokine production, an effect described first for TNF-α and interleukin-1, and later observed for other cytokines including chemokines. As it is not known whether Epo influences the hypoxia-induced microglia response, one of the aims of this study was to investigate the role of Epo in the activation of microglial cells. Because of the role of hypoxia in pro-inflammatory mechanisms in the nervous system, we further assessed the possible protective action of Epo on neuronal cells affected by a pro-inflammatory environment.

Results

Microglial cell activation by hypoxia

The murine microglial cell line EOC-2 was activated by cobalt chloride ($CoCl_2$)-induced chemical hypoxia, and cell activation was characterized by measurement of cell proliferation and the levels of pro-inflammatory mediators such as NO and TNF-α.

As shown in Fig. 1, chemical hypoxia led to significantly increased TNF-α secretion and nitrite production 24 or 48 h after cell activation, while expression of the inducible isoform of nitric oxide synthase (iNOS) was up-regulated. In addition, oxidative stress was induced as indicated by significantly increased ROS levels detected after cell activation (Fig. 1B).

We also investigated whether hypoxia affect microglial cell proliferation. The results of cell counting and MTT assays show a significantly higher EOC-2 proliferation rate compared with controls after exposure to $CoCl_2$ (Fig. 1C).

Erythropoietin effect on microglial cell activation by chemical hypoxia

Although it has been suggested that Epo may be involved in neuroinflammation [10,20,21], its role in microglia remains to be clarified. Therefore, it was interesting to investigate whether Epo affects microglial cell activation. We observed expression of the Epo receptor (EpoR) in the microglial EOC-2 cell line by western blotting (Fig. 2A) and immunofluorescence microscopy (data not shown), which demonstrated the existence of EpoR in both non-activated and $CoCl_2$-treated cells.

As activation of microglial cells may lead to inflammation, we next investigated whether Epo acts as an anti-inflammatory factor, affecting the activation of EOC-2 cells. As shown in Fig. 2B,C, Epo was unable to prevent the increase in nitrite levels and iNOS expression induced by chemical hypoxia. It should be noted that Epo itself induced nitrite production and iNOS up-regulation. Based on previous evidence implicating PI3K in the induction of iNOS [22], we further investigated this signaling pathway. We found that inhibition of the PI3K-mediated pathway by Ly294002

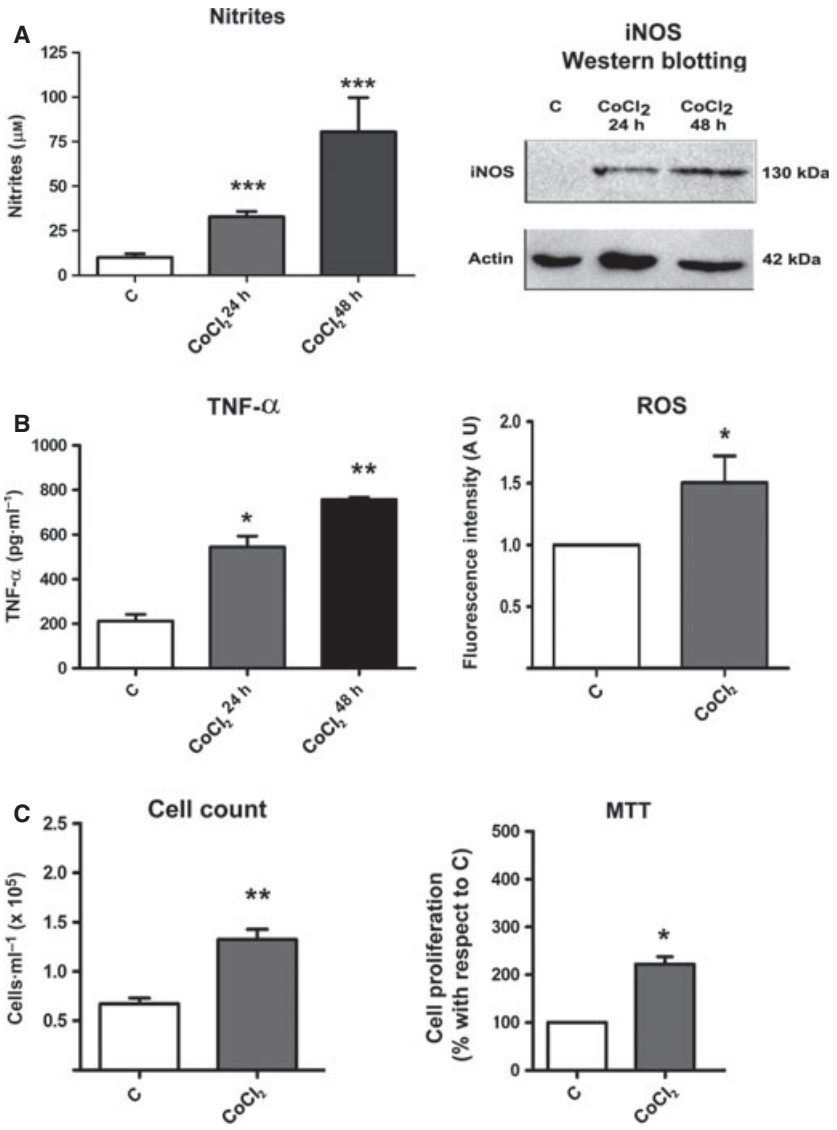

Fig. 1. Microglial cell activation by chemical hypoxia. EOC-2 cultures were exposed or not (control) to chemical hypoxia using $CoCl_2$, and parameters of microglial cell activation were determined. (A, B) Nitrite production (Griess method) and TNF-α secretion (ELISA test) were measured after 24 or 48 h of cell activation. ROS levels were analyzed by fluorescence microscopy using the DCFH-DA probe after 2 h exposure to chemical hypoxia. Significant increases in nitrite (A), TNF-α and ROS (B) were induced by 100 μM $CoCl_2$ treatment. Asterisks indicate statistically significant differences: nitrite, $***P < 0.001$ with respect to control, $n = 6$; TNF-α and ROS, $*P < 0.05$ or $**P < 0.01$ with respect to control, $n = 3$. iNOS expression (A), analyzed by western blotting, increased during the hypoxia treatment. (C) Cell proliferation, analyzed by cell counting (trypan blue exclusion test) and MTT assay, significantly increased after 24 h of chemical hypoxia ($*P < 0.05$, $**P < 0.01$ with respect to control, $n = 7$). The MTT values are expressed as a percentage of the control. Values are means ± SEM of n independent trials.

prevented the production of nitrites by Epo, thus showing another important role for PI3K in Epo-mediated action. As shown in Fig. 2D, Epo was also unable to prevent TNF-α secretion. Interestingly, although Epo did not show a direct anti-inflammatory action in this model, the hormone did have an antioxidant effect as it completely prevented the increase in ROS caused by $CoCl_2$-induced hypoxia (Fig. 2E).

Taking into account this antioxidant effect of Epo and reports describing a relationship between ROS production and microglia proliferation [23], we decided to investigate whether Epo had any effect on cell growth induced by chemical hypoxia. We found that Epo abrogated the increase in EOC-2 cell growth induced by $CoCl_2$ (Fig. 3B). In accordance, prevention of increase in the number of cells positive for the cell proliferation and activation marker PCNA was also observed in microglia cultures pre-treated with Epo (Fig. 3A). As oxidative stress modulates microglial cell proliferation [23], the antioxidant ability of Epo may explain, at least in part, its inhibitory effect on the proliferative response of activated microglia. This assumption is supported by the fact that, under similar conditions, the antioxidant *N*-acetyl cysteine also prevented the mitogenic activity induced by chemical hypoxia (Fig. 3B).

Erythropoietin effect on neurotoxicity induced by activated microglia

The results of this study show that activated microglial cells enhance the pro-inflammatory responses and

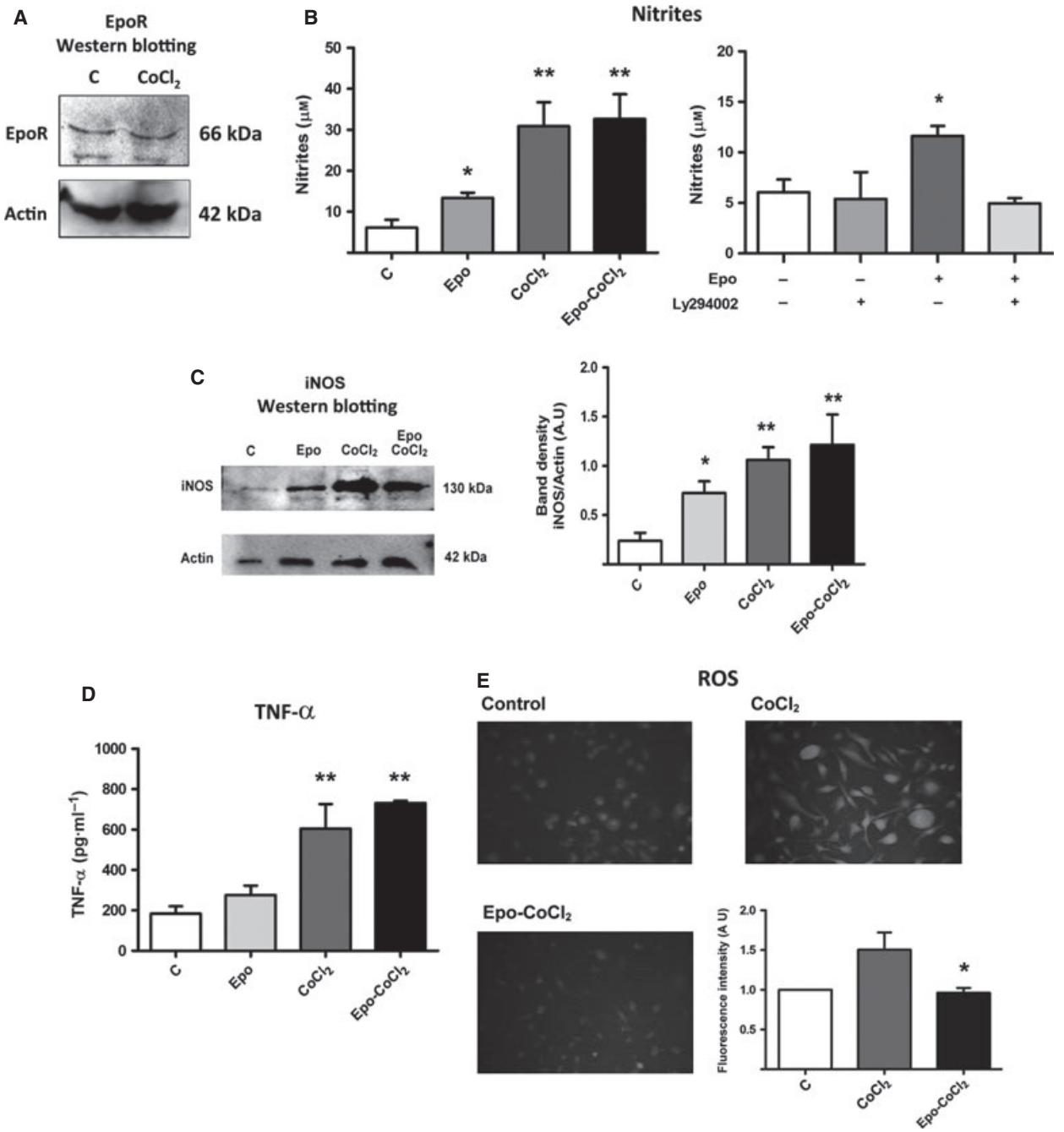

Fig. 2. Erythropoietin action on activated microglial cells. (A) EpoR was detected in the EOC-2 cell line by western blotting of 100 μM $CoCl_2$-treated and untreated cells. (B, C) Nitrite production: EOC-2 cell cultures were incubated with Epo (25 $U \cdot mL^{-1}$) and then exposed or not to 100 μM $CoCl_2$. The increased nitrite secretion and iNOS expression induced by chemical hypoxia was not prevented by the presence of Epo. In fact, Epo induced nitrites, and this effect was prevented by incubation of cells with 25 μM Ly294002 for 2 h before exposure to Epo. The blot is representative of three assays with similar results. Values are means ± SEM of band density with respect to β-actin. Asterisks indicate statistically significant differences: $CoCl_2$ or Epo-$CoCl_2$ versus control, $^{**}P < 0.01$; Epo versus control, $^{*}P < 0.05$; $CoCl_2$ versus Epo-$CoCl_2$, not significant, $n = 5$; Ly294002-Epo versus control, not significant, $n = 3$. (D) Induction of TNF-α expression by chemical hypoxia was not overcome by Epo: $CoCl_2$ or Epo-$CoCl_2$ versus control, $^{**}P < 0.01$. Epo did not induce TNF-α production: Epo versus control or $CoCl_2$ versus Epo-$CoCl_2$, not significant, $n = 3$. (E) ROS levels induced by chemical hypoxia ($CoCl_2$, 2 h) were significantly decreased by 24 h Epo pre-treatment (Epo-$CoCl_2$ versus $CoCl_2$, $^{*}P < 0.05$; Epo-$CoCl_2$ versus control, not significant, $n = 3$). Photographs show positive fluorescence in $CoCl_2$-treated cells but not in control and in Epo-$CoCl_2$ samples. Values are fluorescence intensity/cell area ratio expressed in arbitrary units with respect to control ($n = 3$ independent assays).

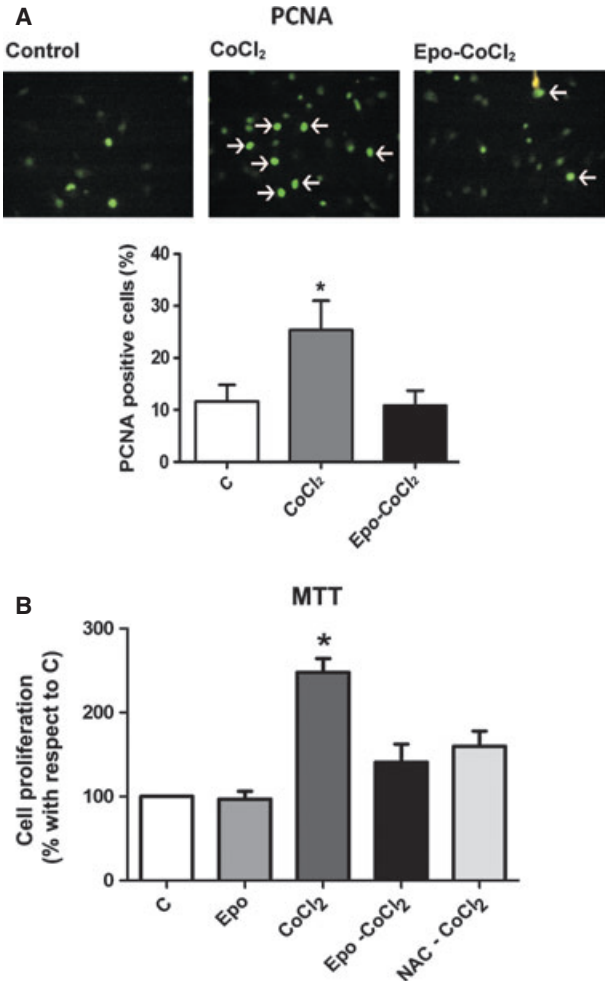

Fig. 3. Effect of erythropoietin on microglial cell proliferation induced by chemical hypoxia. EOC-2 microglial cells were cultured in the presence of Epo (25 U·mL^{-1}, 24 h) and then exposed to 100 μM $CoCl_2$ (6 h). Expression of the proliferation marker PCNA (arrows) was detected by immunofluorescence microscopy (A), and cell proliferation was measured by the MTT assay and expressed as a percentage of the control (B). Pre-treatment with Epo prevented an increase in PCNA expression (*$P < 0.05$ with respect to control or Epo-$CoCl_2$, $n = 3$) as well as induction of cell proliferation by chemical hypoxia (*$P < 0.05$ with respect to control or Epo-$CoCl_2$, $n = 7$). Similar results were observed when EOC-2 cultures were treated with *N*-acetyl cysteine (NAC) before being exposed to chemical hypoxia (*$P < 0.05$ with respect to control; Epo-$CoCl_2$ versus NAC-$CoCl_2$, not significant, $n = 3$). Values are means ± SEM of *n* independent trials.

oxidative stress that may cause neuronal injury. Therefore, further study focused on the cell survival of SH-SY5Y neuronal cultures exposed to a pro-inflammatory environment. Cultures of neuronal cells were performed in the presence of conditioned medium (CM) from EOC–2 cells activated by chemical hypoxia or from RAW 264.7 macrophages induced by lipopolysaccharide (LPS) and interferon-γ (IFNγ). Similar data regarding cell activation (nitrite production and TNF-α secretion) were observed for CM from EOC–2 activated by chemical hypoxia and RAW cultures activated with LPS and IFNγ (data not shown). These *in vitro* pro-inflammatory models showed neuronal cell death by apoptosis. Figure 4 shows that SH-SY5Y cells developed apoptosis when cultured in the presence of CM obtained after 48 h incubation of EOC-2 cells with $CoCl_2$ or after 24 h activation of RAW 264.7 with LPS and IFNγ.

Based on these results, and given the neuroprotective ability of Epo, we further investigated whether this factor was able to protect neuronal cells exposed to the pro-inflammatory environment. It was found that cell pre-treatment with Epo prevented apoptosis of neuronal SH-SY5Y cells exposed to CM from activated cells (Fig. 4). This Epo neuroprotective action against CM from activated EOC-2 cells was mediated by EpoR activation, as such cell protection did not occur when the neuronal cells were previously treated with Ly294002, which inhibits PI3K, kinase involved in the signaling pathway of EpoR activation (Fig. 4C).

Finally, we analyzed whether CM from microglia treated with Epo before activation by chemical hypoxia has any effect on SH-SY5Y neuroprotection. EOC-2 cells were pre-treated with Epo and then activated by $CoCl_2$ to obtain CM. SH-SY5Y cells were cultured in the presence of this Epo-pre-treated CM, and evaluation of cell apoptosis was performed after 48 h. In contrast with CM from activated EOC-2, CM from Epo-pre-treated microglia did not cause apoptosis of SH-SY5Y neuroblastoma cells (Fig. 5).

Discussion

Experimental evidence indicates that microglial cells have dual effects. Under certain circumstances, these cells may be beneficial and may limit neuronal injury in the presence of pathogens and toxic agents. On the other hand, alteration of the natural innate immune response by microglia has direct effects with regard to exacerbating the damage following acute injury to neurons. In recent years, accumulating data have supported the notion that microglia activation, as an indicator of inflammation, is not pro- or anti-neurogenic in itself, but the net outcome is dependent on the balance between secreted molecules with pro- or anti-inflammatory actions [24].

In the present paper, activation of EOC-2 microglial cells was achieved by hypoxic conditions induced by the presence of cobalt chloride. This compound is used to study the hypoxic signaling pathway because of its

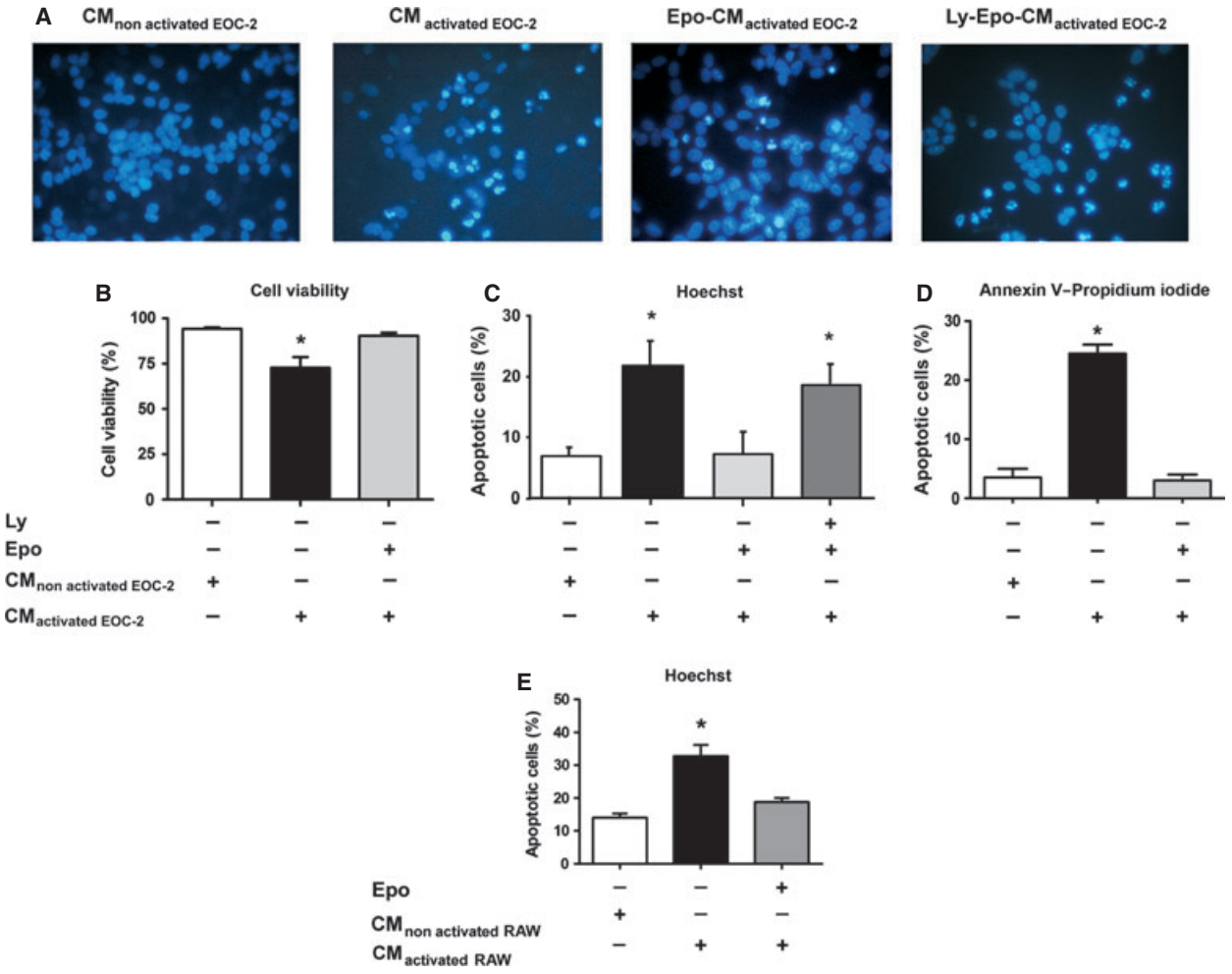

Fig. 4. Effect of erythropoietin on neuronal cells exposed to conditioned medium from activated microglia or macrophages. Microglial conditioned medium (CM activated EOC-2) was obtained after activation of EOC-2 cells by chemical hypoxia (100 μM $CoCl_2$, 48 h). This CM and CM from control cells (CM non activated EOC-2) were added to 48 h cultures of neuronal SH-SY5Y cells. To study Epo neuroprotection, the SH-SY5Y cells were exposed to 25 U·mL^{-1} Epo for 24 h before CM addition. (A) Representative photomicrographs (Hoechst images) for the various treatments. (B–D) Cell viability was analyzed by cell counting by the trypan blue exclusion test (B) and apoptosis was analyzed by fluorescence microscopy using Hoechst (C) or Annexin V/propidium iodide staining (D). Values are means ± SEM for the percentage of apoptotic cells relative to the total cell number. Asterisks indicate statistically significant differences (*$P < 0.05$) for assays cultured under inflammatory conditions versus controls and Epo-protected cultures ($n = 5$ independent assays). In inhibition assays, SH-SY5Y cells received a 2 h pre-treatment with 25 μM Ly294002 (PI3K inhibitor) before addition of Epo and/or CM (C). The protective effect of Epo was completely inhibited (Ly-Epo-$CM_{activated\ EOC\text{-}2}$ versus Epo-$CM_{activated\ EOC\text{-}2}$, *$P < 0.05$; Ly-Epo-$CM_{activated\ EOC\text{-}2}$ versus $CM_{activated\ EOC\text{-}2}$, not significant; $n = 3$). (E) SH-SY5Y cells, pre-treated or not with Epo (25 U·mL^{-1}, 24 h), were cultured for additional 24 h in the presence of CM obtained from RAW 264.7 macrophages activated by 10 ng·mL^{-1} LPS + 100 U·mL^{-1} IFNγ. Apoptosis was analyzed by fluorescence microscopy using Hoechst stain. The asterisk indicates a statistically significant difference (*$P < 0.05$) with respect to controls (cultures with CM from non-activated macrophages); the P value for Epo-protected cultures with respect to control was not significant ($n = 3$ independent trials).

hypoxia-mimicking effect under normoxic conditions. Like hypoxia, $CoCl_2$ activates hypoxia-inducible factor 1α, which stimulates the transcription of several genes that are associated with hypoxia [25,26].

Cellular exposure to hypoxia not only induced an increase in nitrite and TNF-α secretion but also affected cell proliferation (Fig. 1). These results are in accordance with those of Mander *et al.* [23], who reported that hypoxia is a potent stimulator of microglia proliferation. A similar mitogenic effect was also detected after cell activation under inflammatory conditions [27–29].

We also observed induction of iNOS expression and increased ROS production after microglia activation

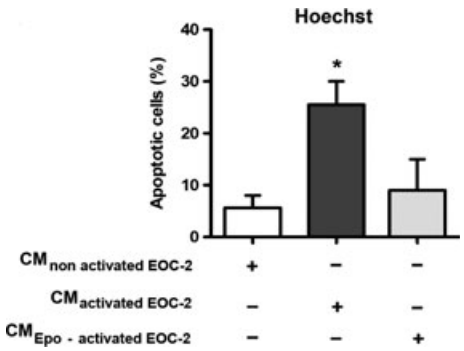

Fig. 5. Erythropoietin neuroprotection by indirect action on microglia activation. Microglial EOC-2 cells were pre-treated with Epo (25 U·mL^{-1}, 24 h) and then activated for 48 h by chemical hypoxia induced by 100 μM $CoCl_2$. At the end of this period, CM were obtained and added to SH-SY5Y cell cultures. Cell apoptosis was evaluated by fluorescence microscopy after Hoechst staining. The results show that CM from activated EOC-2 cells induced neuronal cell death, but CM from microglial cell cultures pre-treated with Epo was unable to induce apoptosis of the SH-SY5Y neuroblastoma cells (*$P < 0.05$ versus cultures in CM from activated EOC-2 cells, $n = 3$ independent trials).

by hypoxia (Fig. 1). In accordance, Lu *et al.* [22] have reported NO production accompanied by iNOS induction during hypoxia of either primary rat microglial cultures or the established microglial cell line BV-2, indicating that hypoxia leads to inflammatory activation of microglia. With regard to the oxidative environment, it has been reported that ROS may enhance the microglia inflammatory response [23]. These authors demonstrated that several pro-inflammatory mediators are able to directly stimulate microglial NADPH oxidase, leading to subsequent hydrogen peroxide production and inducing microglia proliferation. The redox imbalance amplifies the inflammatory reaction. At the same time, this increase activates antioxidant genes in order to restore the redox balance, driving active microglia back to the resting state [30].

Taking into account the role that microglia may play on either progression or amelioration of brain damage, agents that modulate microglial cell activation are an important area of study.

It is now recognized that Epo is not limited to support erythropoietic progenitor survival and development, as many studies have linked Epo to potential anti-apoptotic or anti-inflammatory effects in non-hematopoietic tissues. However, to date, no clear information is available about possible effect of Epo in the interaction between microglia and neurons.

As specific receptors for Epo are present in the murine EOC-2 cell line (Fig. 2), we further investigated whether Epo affects microglial cell activation. Cell cultures were incubated in the presence of Epo before being activated by $CoCl_2$, and, under these conditions, Epo was unable to inhibit the hypoxia-induced cell activation as determined by secretion of nitrites and TNF-α. Instead, we observed that Epo induced nitrite production. The lack of ability of Epo to induce nitrites when PI3K was inhibited confirms the involvement of the Epo/EpoR signaling pathway (Fig. 2). Similar Epo action on NO secretion in various tissues has been reported previously. Epo stimulation up-regulated endothelial nitric oxide synthase activity and enhanced NO production [31,32]. Both Epo alone and combined with interleukin-1β induced significant production of nitrite in fibroblast-like cell culture supernatants, which may be mediated by NOS activation [33].

Several studies have obtained conflicting data regarding Epo and inflammation. Epo showed an anti-apoptotic action without affecting the pro-inflammatory function of rat microglial cells [21,34]. However, in peritoneal macrophages, Epo showed a pro-inflammatory effect by inducing increased nitrite and TNF-α production [35]. In contrast with this finding, Epo inhibited the induction of pro-inflammatory genes, including TNF-α and inducible NO synthase, in activated macrophages, which was mechanistically attributable to blockage of nuclear factor NF-κB activation by Epo [36]. Taking together, these results suggest that the effects of Epo may be highly dependent on tissue target and factors involved in the inflammatory condition.

With regard to the ability of Epo to maintain microglial cell integrity, Li *et al.* [19] demonstrated that Epo significantly increased microglial cell survival during oxygen/glucose deprivation. The results of the present paper suggest that, although Epo does not exert a direct anti-inflammatory action on activated EOC-2 cells, it prevented the increase in cell proliferation induced by chemical hypoxia (Fig. 3). This is in accordance with results reported by Chong *et al.*, who observed that administration of Epo prior to NO exposure prevented PCNA expression in microglia from the rat cerebral cortex [20]. It may be speculated that the inhibitory effect of Epo on microglia proliferation may ameliorate the long-term effect of microglia activation.

In the present study, Epo demonstrated its known antioxidant capacity during microglia activation as pre-treatment of cells with Epo prevented ROS production induced by $CoCl_2$. This Epo antioxidant effect has also been found in other models, such as cardiomiocyte, endothelial or PC-12 cell cultures [37,38].

A non-specific antioxidative effect of Epo cannot be ruled out. In this regard, Chattopadhyay *et al.* [39] proposed that EPO protects red cell membranes from lipid peroxidation by scavenging hydroxyl radicals. However, irrespective of the mechanisms involved, the

Epo antioxidant action observed in the present study may be related, at least in part, to the prevention of microglia proliferation induced by hypoxia. This is supported by the findings of Mander *et al.* [23], who demonstrated that microglia proliferation in response to interleukin-1 or TNF-α was mediated by hydrogen peroxide released by NADPH oxidase.

The signs of apoptosis in neuronal SH-SY5Y cells induced by CM from activated microglia or macrophages in the present study appeared to be similar to those induced by various agents, such as staurosporine, TNF-α or hypoxia, as previously observed in our laboratory [14,17,18]. In those studies, we demonstrated the ability of Epo to overcome the direct effects of pro-apoptotic agents on neuronal cells through activation of the Janus kinase 2 and PI3K signaling pathways. In this paper, we observed neuroprotection by Epo in cells exposed to CM acting as pro-inflammatory microenvironment. An indirect indication that the EpoR mediates this action is obtained by inhibition of the anti-apoptotic effect of Epo on neuronal cells in experiments with Ly294002, the inhibitor of PI3K, a major pathway downstream of EpoR activation (Fig. 4).

Another possible explanation stems from the recent elucidated molecular mechanism of Epo protective action [40]. A number of genes, particularly *Egr2*, were up-regulated, or their induction by ischemia was prolonged by Epo. Members of the Egr2 family mediate cognitive functions associated with neuronal plasticity. In their discussion, Mengozzi *et al.* speculate that neuroinflammation may be a factor that primes the brain for the tissue-protective action of Epo. Thus, Epo may prevent apoptosis even though it is not able to reduce inflammatory cytokines.

On the other hand, the damaging effects of ROS on neurons are well known and may be overcome by use of antioxidative drugs. As suppression of formation of NO-mediated free radicals has been proposed to account for the cytoprotective effect of Epo [41], we suggest that, in addition to the well-known anti-apoptotic mechanisms, the effect of Epo as an antioxidant agent may contribute to explaining the present results. It has been reported that, during activation of microglia, peroxynitrite (ONOO–) is produced by interaction between NO and ROS. It was also suggested that this compound, which is very toxic to neuronal cells, may be removed by ROS scavengers [42]. The antioxidant action of Epo during microglia activation may produce a less favorable environment for the development of free radicals (Fig. 2), thus having a protective effect against neuronal damage. Based on these results, we analyzed the impact of Epo on the survival of neurons after exposure to CM from microglial cells pre-treated with Epo before activation by chemical hypoxia. We found that neuronal SH-SY5Y cells showed apoptosis in CM from activated microglia but were able to survive when cultured in CM from microglial cells pre-treated with Epo before cell activation, suggesting that Epo modulates the final effect of microglia activation on neuronal fate (Fig. 5).

In conclusion, using *in vitro* models, we not only show new effects of Epo on activation of microglia, but also provide hitherto unknown information regarding protection of neuronal cells. Epo maintained the survival of neuronal SH-SY5Y cells exposed to a pro-inflammatory environment. The Epo action on activated EOC-2 microglial cells prevented generation of ROS. In addition, Epo increases the resistance of neurons to subsequent damage derived from the pro-inflammatory function of microglial and macrophage cells (Fig. 6). Therefore, we suggest that, in addition to the direct anti-apoptotic action of Epo on neuronal cells, its antioxidant ability may have an indirect influence on neuronal survival by modulation of the pro-inflammatory environment.

Altogether, the results suggest that Epo exerts neuroprotection independently of the presence of pro-inflammatory cytokines, a fact that underlines the multi-functional role of Epo as well as its potential clinical application as a cytoprotective agent.

Experimental procedures

Reagents and antibodies

All chemicals used were of analytical grade. Dulbecco's modified Eagle's medium, RPMI-1640 medium, and

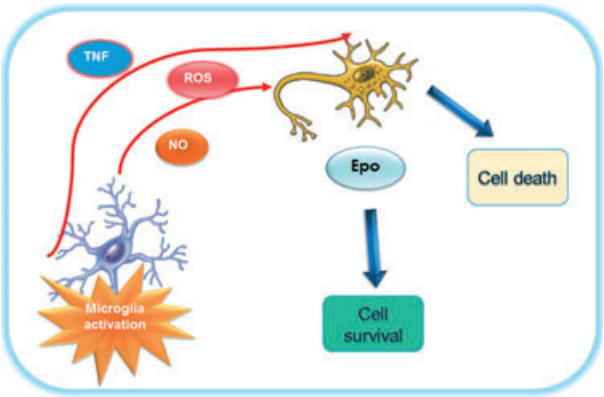

Fig. 6. Erythropoietin neuoprotection. Neuronal cells cultured in the presence of conditioned medium from activated microglia developed significant apoptosis induced by high levels of NO, TNF-α and ROS, an effect that was prevented by Epo.

Ham's F-12 culture medium were obtained from Gibco (Life Technologies, Carlsbad, CA, USA) and goat anti-rabbit IgG (H + L) Alexa fluor 488 was from Molecular Probes (Life Technologies). Antibody against PCNA was obtained from Chemicon Internacional (Temecula, CA, USA) and antibodies against actin and erythropoietin receptor (EpoR, M20) were obtained from Santa Cruz Biotechnology (Dallas, TX, USA). EDTA, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), L-glutamine, 2,7-dichlorofluorescin diacetate (DCFH-DA), *N*-acetyl cysteine, Hoechst 33258 dye, and p-formaldehyde were obtained from Sigma-Aldrich (St. Louis, MO, USA). Ly294002 was obtained from Calbiochem (Billerica, MA, USA) and Chemiluminiscent system (ECL) and nitrocellulose membranes (Hybond-ECL) were from GE Healthcare Lifesciences (Piscataway, NJ, USA). IFN gamma, Annexin V–FITC apoptosis detection kit II, recombinant human TNF-alpha, TNF-ELISA kit, and antibody against iNOS were obtained from BD Biosciences (San Jose, CA, USA). Sodium dodecylsulfate, acrylamide/bis-acrylamide solution, Triton X-100, Tween-20, cobalt chloride, and dimethylsulfoxide were obtained from Merck (Darmstadt, Germany). Fetal bovine serum and penicillin/streptomycin were obtained from PAA Laboratories GmbH (Cölbe, Germany). Recombinant human erythropoietin was kindly supplied by Zelltek (Santa Fe, Pcia. de Santa Fe, Argentina).

Cell cultures

EOC-2 microglial cells (murine, CRL-2467; American Type Culture Collection, Manassas, VA, USA) were maintained in 25 cm^2 plastic tissue-culture flasks (Falcon BD, Franklin Lake, NJ, USA) containing Dulbecco's modified Eagle's medium supplemented with 2 mM L-glutamine, antibiotics (100 $U \cdot mL^{-1}$ penicillin and 100 $\mu g \cdot mL^{-1}$ streptomycin), 10% v/v fetal bovine serum and 20% conditioned medium from LADMAC cells (American Type Culture Collection), which contains colony stimulating factor-1 secreted by this cell line [43].

RAW 264.7 macrophage progenitor cells (murine, TIB-71; American Type Culture Collection) were maintained in RPMI-1640 supplemented with 10% v/v fetal bovine serum and antibiotics (100 $U \cdot mL^{-1}$ penicillin and 100 $\mu g \cdot mL^{-1}$ streptomycin).

SH-SY5Y neuroblastoma cells (human, CRL-2266, American Type Culture Collection) were maintained in 1 : 1 Dulbecco's modified Eagle's medium/Ham's F-12 medium supplemented with 2 mM L-glutamine, antibiotics (100 $U \cdot mL^{-1}$ penicillin and 100 $\mu g \cdot mL^{-1}$ streptomycin) and 10% v/v heat-inactivated fetal bovine serum.

All cultures were performed at 37 °C with 5% CO_2 and 100% humidity. The medium was replaced every 2 days, and cells were divided into separate flasks before they reached confluence [18].

Experimental treatments

Microglia activation

EOC-2 cells were seeded in 35 mm Petri dishes, grown to 80% confluence, and then activated by various concentrations (50–200 μM) of the hypoxia-mimicking agent cobalt chloride ($CoCl_2$), with 100 μM being the concentration chosen for experiments with activated microglia. EOC-2 cell activation was performed for 24 or 48 h, as indicated in the figure legends.

RAW 264.7 activation

Cells were activated by exposure to 10 $ng \cdot mL^{-1}$ lipopolysaccharide (LPS) and 100 $U \cdot mL^{-1}$ IFNγ for 24 h.

Neuronal cytotoxicity induced by activated microglia or macrophages

Activation of EOC-2 microglial cells (48 h) or RAW 264.7 cells (24 h) was performed as deacribed above. After centrifugation (350 ***g***, 8 °C, 5 min), 0.3 mL of cell-free supernatant (CM, conditioned medium) were transferred to wells containing SH-SY5Y cells. The neuroblastoma cell cultures (2×10^5 $cells \cdot mL^{-1}$) were previously plated for 72 h on 0.5 mL of Dulbecco's modified Eagle's medium/Ham's F-12 medium containing 10% fetal bovine serum. After 48 h incubation of SH-SY5Y cells with CM, evaluation of neuronal cell survival was performed.

Culture medium incubated for 48 h with $CoCl_2$ or LPS plus IFNγ at concentrations used for cell activation was tested on SH-SY5Y cultures in order to analyze cytotoxicity. According to results of the MTT assay and cell counting by the trypan blue exclusion test, there was no alteration of cell viability.

Assessment of cell viability/proliferation

Cell counting and viability determination in samples stained with trypan blue (4 $g \cdot L^{-1}$ in 9 $g \cdot L^{-1}$ NaCl) were performed in a Neubauer chamber by microscopy (Nikon YS100, Tokyo, Japan).

The MTT reduction assay was used to assess cell viability/proliferation [18]. Cells were cultured in 24-well plates at a density of 0.5×10^5 $cells \cdot mL^{-1}$ and subjected to the appropriate treatments. After removal of the medium, cells were washed with isotonic phosphate-buffered saline (0.137 M NaCl, 3 mM KCl, 1.9 mM NaH_2PO_4, 8.1 mM Na_2HPO_4, pH 7.4) and incubated for 4 h at 37 °C with MTT (0.5 $mg \cdot mL^{-1}$ final concentration). After centrifugation (13 000 ***g***, 8 °C, 10 min) the supernatant was removed, and the pellet was washed with isotonic phosphate-buffered saline (see above). Finally, 100 μL of 0.04 M HCl in isopropanol was added to dissolve the blue formazan product

(reduced MTT), and centrifuged (13 000 ***g***, 8 °C, 10 min). The supernatant was quantified by measuring the absorbance at a test wavelength of 570 nm and a reference wavelength of 655 nm using a microplate reader (Bio-Rad Laboratories, Hercules, CA, USA).

Measurement of nitric oxide

Nitric oxide (NO) production was determined by measuring the accumulation of nitrite, the stable metabolite of NO, in culture medium. Isolated supernatants collected from cultures containing 1×10^5 microglial cells exposed to chemical hypoxia for the indicated periods were mixed with equal volumes of Griess reagent (1% sulfanilamide, 0.1% naphthylethylenediamine dihydrochloride and 2% phosphoric acid), and incubated at 25 °C for 10 min. Absorbance at 540 nm was measured using a microplate reader.

Cytokine quantification

TNF-α was measured by ELISA in supernatants obtained as described above for NO measurement. The ELISA test was performed according to the manufacturer's instructions. In each experiment, samples were analyzed in duplicate against standards of known concentration.

Western blot analysis

After treatments, culture medium was discarded and cells were lysed with Laemmli buffer (4% SDS, 20% glycerol, 10% 2-mercaptoethanol, 0.004% bromophenol blue, 0.125 M Tris/HCl, pH 6.8). Lysates were boiled for 3 min, resolved by 10% SDS/PAGE using Tris-glycine pH 8.3 (25 mM Tris, 192 mM glycine, 0.1% SDS) as running buffer, and then electroblotted onto a Hybond nitrocellulose membrane (transfer buffer: 25 mM Tris, 195 mM glycine, 0.05% SDS, pH 8.3, and 20% v/v methanol). Non-specific binding sites on the membrane were blocked (1 h, 25 °C) using 5% ECL membrane blocking agent in Tris-buffered saline (25 mM Tris, 137 mM NaCl, 3 mM KCl, pH 7.4) containing 0.1% Tween-20 (TBS-Tween). Blots were briefly rinsed with two changes of TBS-Tween buffer, and then washed three times for 10 min each. Afterwards, blots were incubated with an appropriate dilution of specific antibodies (1 : 500 anti-iNOS, 1 : 200 anti-EpoR or 1 : 200 anti-actin antibodies) for 1 h at 4 °C, washed three times for 10 min each with TBS-Tween, and probed using a 1 : 1000 dilution of either anti-mouse or anti-rabbit horseradish peroxidase-conjugated antibodies for 1 h at 25 °C [44]. After washing, blots were incubated with the enhanced chemiluminiscence substrate (ECL kit) and the bands were detected using Fujifilm Intelligent Dark Box II equipment coupled to an LAS-1000 digital camera (Valhalla, NY, USA). IMAGE READER LAS-1000 and LPROCESS V1.Z2 software were used to visualize the bands.

Fluorescence microscopy

Reactive oxygen species

Cells were cultured on slide covers plated on a 24-well plate. After treatment, cells were incubated with 100 μM DCFH-DA for 30 min, washed with isotonic phosphate-buffered saline (see above), and fixed with acetic acid/methanol (1 : 3) at 25 °C for 10 min. Finally, samples were washed twice with isotonic phosphate-buffered saline (see above), and mounted with 50% v/v glycerol in isotonic phosphate-buffered saline (see above). For fluorescence microscopy observation, Axiovert 135 (Zeiss, Oberkochen, Germany) equipment was used under UV illumination at 365 nm. A Canon Power Shot G9 camera (Lake Success, NY, USA) and AXIOVISION version 4.6 software were used for image acquisition. For each sample, 10 digital images were analyzed using IMAGEJ software (http://imagej.nih.gov/ij/), and the DCFH-DA mean fluorescence intensity/cell area ratio was calculated and expressed as arbitrary units. The results shown are standardized with respect to cell proliferation.

Proliferating cell nuclear antigen

For microglia activation, PCNA expression was analyzed by immunofluorescence with antibody against PCNA (1 : 80 dilution, 60 min, 37 °C), visualized with Alexa Fluor 488 dye-labeled secondary antibody (60 min, 37 °C), and nuclei were stained with 0.05 $g \cdot L^{-1}$ Hoechst 33258 dye (30 min, 25 °C). After washing three times with isotonic phosphate-buffered saline (see above), samples were mounted using 50% v/v glycerol in isotonic phosphate-buffered saline (see above). Images were acquired under UV illumination at 365 nm in an Axiovert 135 microscope (Zeiss), with a Canon Power Shot G9 camera and AXIOVISION version 4.6 software. Ten images were obtained by random sampling, and analysis was performed using IMAGEJ software. At least 400 total cells were counted. The results are expressed as percentage of PCNA-positive cells with respect to total cell number. The appropriate isotype antibody was used as negative control.

Annexin V/propidium iodide analyses

At the end of the experiment, SH-SY5Y cells were washed with binding buffer and then incubated with Annexin V and propidium iodide (15 min at room temperature), according to the manufacturer's indications. After this period, samples were washed with binding buffer, mounted and analyzed by immunofluorescence microscopy. A differential cell count for Annexin V and propidium iodide was performed by evaluating at least 300 cells.

Hoechst nuclear staining of apoptotic cells

SH-SY5Y cell cultures were developed on slide covers plated in 35 mm Petri dishes. Four slides per experimental condition and controls were run simultaneously. After treatments, cells were fixed with 4% v/v *p*-formaldehyde in isotonic phosphate-buffered saline (see above) solution for 20 min at 4 °C, exposed to 0.05 $g \cdot L^{-1}$ Hoechst 33258 dye in isotonic phosphate-buffered saline (see above) for 30 min at room temperature, and washed three times with isotonic phosphate-buffered saline (see above). Finally, samples were mounted, and fluorescent nuclei with apoptotic characteristics were detected and analyzed by immunofluorescence microscopy. Apoptotic cells were identified by morphology and nuclear fluorescence intensity. The condensed chromatin within apoptotic cells stains particularly heavily, showing blue fluorescence. In addition, small apoptotic bodies released from nuclei are also detected because of their brilliant blue color. A differential cell count was performed by evaluating at least 400 cells [44].

Double-blind studies were performed in all fluorescence assays.

Statistical analysis

Results are expressed as mean and standard error (SEM) of *n* independent trials (at least three) as indicated in each figure. Kruskal-Wallis one-way analysis of variance was used to determine differences among more than two groups. Groups having significant differences in the variance analysis were then evaluated with a non-parametric Mann-Whitney U test. A P values < 0.05 was considered to indicate statistical significance.

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