Erythropoietin reduces cisplatin-induced neurotoxicity without impairment of cytotoxic effects against tumor cells

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Abstract. Cisplatin, a widely used chemotherapeutic is approved for the management of various solid tumors. Administration of cisplatin is associated with induction of significant toxicities that include neurotoxicity and nephrotoxicity, the latter leading to severe and debilitating anemia. Since erythropoietin, a hematopoietic growth factor that corrects chemotherapy-induced anemia, reduces transfusion requirements and seems to improve the patient's quality of life, has been shown to exert cytoprotective effects we decided to investigate its direct influence on cisplatin-induced neurotoxicity against primary cortical neurons isolated from rats. We observed that pre-treatment of neurons with erythropoietin significantly protects these cells from cisplatin-induced cytotoxicity. These effects correlated with amelioration of cisplatin-mediated activation of ERK1/2 kinases and decreased cleavage of caspase 3. Similarly to erythropoietin, a selective ERK1/2 inhibitor significantly reduced cisplatin-induced cytotoxicity against neuronal cells. Importantly, using the same experimental setting we did not observe any protection from cisplatin cytotoxicity against four established tumor cell lines. Altogether our studies confirm that erythropoietin might be an effective cytoprotective agent that reduces cisplatin-induced neurotoxicity.

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Abbreviations: CNS, central nervous system; Epo, erythropoietin; FBS, fetal bovine serum; TNF, tumor necrosis factor

Key words: erythropoietin, cisplatin, neurotoxicity

Introduction

Cisplatin is among the most widely used chemotherapeutics approved for the management of germ-cell tumors, advanced bladder carcinoma, adrenal cortex carcinoma, breast cancer, head and neck carcinoma, lung carcinoma and ovarian tumors among others (1). Similarly to other anticancer therapeutics cisplatin treatment is associated with significant toxicities that include neurotoxicity and nephrotoxicity, the latter leading to severe and debilitating anemia (2). Cisplatin-mediated neurotoxicity results from nerve damage and is most prominent in peripheral nerves leading to sensory impairment, pain, ataxia and hearing loss. At least to some extent peripheral neurotoxicity results from accumulation of cisplatin in dorsal root ganglia with their subsequent damage followed by secondary nerve fiber axonopathy (3). Cisplatin-mediated neurotoxicity is a significant limitation to effective anti-cancer treatment as it may lead to dose-reduction or even treatment withdrawal. Therefore, effective strategies to reduce the severity of nerve damage following chemotherapy are intensively being searched for.

Erythropoietin (Epo) is a sialoglycoprotein hormone that stimulates erythrocyte production in the bone marrow (4). Although it is usually referred to as a lineage-specific hematopoietin it can also exhibit significant effects in non-hematopoietic tissues. Epo was shown to act as a tissue-protective cytokine, especially within nervous tissue, kidney and cardiac muscle, and its receptors are widely distributed in a variety of tissues (5). Epo protects neurons from apoptosis in cell culture studies and in animal models of CNS injury (6,7). It can protect neurons from ischemia reperfusion-induced injury (7,8), metabolic stress (9), HIV-induced damage (10) or even mechanical injury such as nerve compression or trauma (11,12). It reduces the severity of diabetic neuropathy in animals (13) and ameliorates nerve function during experimental autoimmune encephalomyelitis (14). Recently, Epo was also shown to protect against cisplatin-mediated peripheral neuropathy (15-17). Numerous studies identified Epo receptors on the surface of a variety of cancer cells (18-21). This has

raised safety concerns about the use of Epo in the treatment of cancer-related anemia (22). In this study we evaluated whether Epo can directly influence the cytotoxic effects of cisplatin against primary rat neurons cultured *in vitro*. We have also studied whether Epo influences cisplatin-induced cytotoxicity against established tumor cell lines.

Materials and methods

Cell culture. Cortical neurons were prepared from newborn Harlan Sprague-Dawley rats and cultured at a density of 1500-2000 cells/well in basal medium Eagle (BME, Sigma, St. Louis, USA). The BME growth medium was composed of (per 100 ml) 86 ml of BME, 10 ml of calf serum (Hyclone, Logan, UT), 1.4 ml of 2.5 M glucose (35 mM final), 0.5 ml of 0.2 M L-glutamine (1 mM final), 0.5 ml of penicillin/streptomycin (Sigma), 0.45 ml of stable vitamin mix (3 mg/ml L-proline, 3 mg/ml L-cystine, 1 mg/ml p-aminobenzoic acid, 0.4 mg/ml vitamin B-12, 2 mg/ml *myo*-inositol, 2 mg/ml choline chloride, 5 mg/ml fumaric acid, 80 μg/ml coenzyme A, 0.4 μg/ml D-biotin, and 0.1 mg/ml DL-6,8,-thioctic acid), 50 μl of ITS (5 mg/ml insulin, 5 mg/ml human transferrin, and 5 μg/ml sodium selenite; Sigma), 0.5 ml of 1.6 mg/ml putrescine (Sigma), 0.5 ml of 5 mg/ml transferrin (Sigma), and 12 μl of 1 mM progesterone. Cytosine arabinoside (Sigma) at a concentration of 2.5 μM was added on the second day *in vitro* after seeding (DIV 2) to inhibit the proliferation of non-neuronal cells. Cells were maintained in a humidified incubator with 6% CO_2 at 37˚C. Plates and Labtek 8-chamber slides (Nunc Nalgene, Naperville, IL) were coated with poly-D-lysine and laminin (Sigma). Tumor cells (MDA-MB231 breast carcinoma, A375 melanoma, MDAH2774 ovarian carcinoma, and Mia PaCa-2 pancreatic carcinoma) were purchased from ATCC (Manassas, VA, USA) and cultured in a humidified incubator with 5% CO_2 at 37˚C in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum, antibiotics, and L-glutamine (2 mM) (all from Invitrogen, Carlsbad, CA) as described (23,24).

Reagents. Human recombined erythropoietin (Epo) (NeoRecormon, kindly provided by F. Hoffman-LaRoche Ltd., Basel, CH) was dissolved in distilled water to the concentration of 5000 U/ml. Cisplatin (Platidiam, Pliva-Lachema, Brno, The Czech Republic) was dissolved in distilled water to the stock concentration of 1 mg/ml. Both drugs were further resuspended in culture medium to the concentrations required. LY294002 (PI3 kinase inhibitor) was purchased from Cell Signaling, SB203580 (p38/MAPK inhibitor) and PD169316 (p38/MAPK inhibitor) were from Calbiochem (La Jolla, CA), and UO126 (ERK1/2 inhibitor) was from Promega (Madison, WI, USA).

MTT assay. The cytostatic/cytotoxic effects were tested in a standard 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as described elsewhere (25). Briefly, the cells were seeded onto a 96-well flat bottom microtiter plate (Nunc) at a density of 1500-2000 cells/well. Epo and cisplatin diluted to appropriate concentrations with culture medium were added to a final volume of 200 μl 4 days (DIV 4) and 5 days (DIV 5) after seeding, respectively. Cells were treated with an indicated concentration of both drugs for 72 h (Epo) and 48 h (cisplatin). MTT solution at a concentration of 1 mg/ml was added to each well for the last 2 h of incubation. Then, the cells were lysed using resuspension buffer containing SDS (0.2 g/ml) and DMF (0.5 ml/ml) at pH 4.7 and left overnight in the incubator. On the following day the plates were read in an ELISA reader (SLT Labinstrument GmbH, Salzburg, Austria) using a 570-nm filter. Cytotoxicity was expressed as relative viability of cells (% of control cultures incubated with medium only) and was calculated as follows: relative viability = (Ae - Ab) x 100/(Ac - Ab), where Ab is the background absorbance, Ae is experimental absorbance, and Ac is the absorbance of untreated controls. A series of independent experiments were performed and the results presented are representative.

Western blotting. For Western blotting primary cortical neurons were cultured with 0.1 U/ml Epo and 10 μg/ml cisplatin for 72 and 48 h, respectively. Drugs were added at day 4 (DIV 4) or day 5 (DIV 5) after seeding. After an indicated time of culture the cells were washed with PBS and lysed with Triton lysis buffer containing 20 mM Tris, 137 mM NaCl, 25 mM ß-glycerophosphate, 2 mM NaPPi, 1% Triton X-100, 10% glycerol, 2 mM benzamidine, 0.5 mM DTT and 2 mM EDTA supplemented with Complete® protease inhibitor cocktail tablets (Roche Diagnostics, Mannheim, Germany). Protein concentration was measured using Bio-Rad protein assay (BioRad, Hercules, CA, USA). Equal amounts of whole cell proteins were separated on 12% SDS-polyacrylamide gel, transferred onto Protran® nitrocellulose membranes (Schleicher and Schuell BioScience Inc., Keene, NH, USA), blocked with TBST [Tris buffered saline (pH 7.4) and 0.05% Tween-20] supplemented with 5% nonfat milk and 5% FBS as described (26). The following antibodies at 1:1000 dilution were used for the 24-h incubation: mouse monoclonal anti-α-tubulin (Promega Corporation, Madison, WI, USA), mouse monoclonal anti-phospho-p44/42 MAPK (Cell Signaling), rabbit polyclonal anti-EpoR (SantaCruz), mouse monoclonal anti-caspase 3 (Cell Signaling), mouse monoclonal anti-phospho-JNK (Cell Signaling), and rabbit polyclonal anti-JNK (Cell Signaling). After extensive washing with TBST, the membranes were incubated for 45 min with corresponding horseradish peroxidase-coupled secondary antibodies (Jackson Immuno Research). The reaction was developed using SuperSignal WestPico Trail Kit® (Pierce). The x-ray film (Kodak) was exposed for 30 sec (anti-α-tubulin) or 5 min (remaining antibodies) and developed with a standard x-ray film developer.

Statistical analysis. Data were calculated using Microsoft™ Excel 2003. Differences in *in vitro* cytotoxicity assays were analyzed for significance by Student's t-test. Significance was defined as a two-sided $P<0.01$.

Results

In the initial experiment we searched for expression of the Epo receptor (EPO-R) in primary cortical neurons prepared from newborn Harlan Sprague-Dawley rats. Using Western blotting we observed a band corresponding to a predicted molecular weight of EPO-R (56-57 kDa) (Fig. 1). The presence

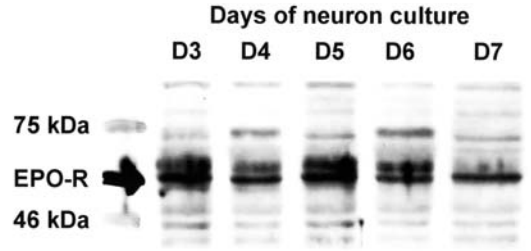

Figure 1. Expression of erythropoietin receptors in primary rat cortical neuron cultures. Cortical neurons were prepared from newborn Harlan Sprague-Dawley rats. On days 3-7 cells were harvested and the prepared cell lysates were subjected to Western blot analysis: 50 μg of protein extracts were separated during electrophoresis.

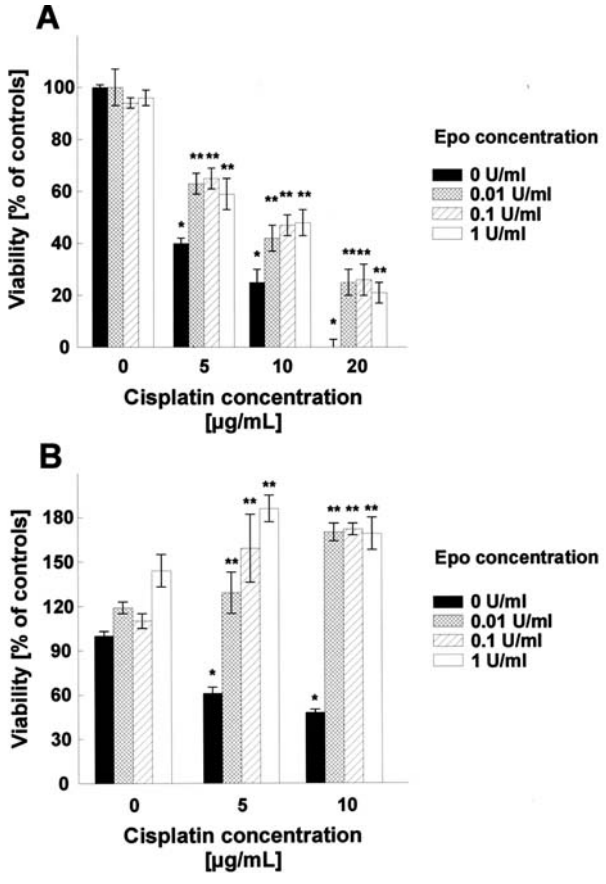

Figure 2. Erythropoietin ameliorated cisplatin-induced cytotoxicity towards rat cortical neurons. Freshly isolated neurons were pre-incubated with either diluents or Epo for 24 h. The incubation with cisplatin followed for another 48 h with or without fresh Epo. The cytostatic/cytotoxic effects were tested with MTT assay. A and B represent results of two independent experiments with different isolates of neurons. Bars represent means ± standard deviations. *P<0.01 as compared with controls, **P<0.01 as compared with cisplatin-treated cells (Student's t-test).

of EPO-R in neurons was monitored during 7 consecutive days of culture, which corresponded to a maximal length of cell culture during further experiments.

Incubation of rat neurons with cisplatin for 48 h resulted in a dose-dependent cytotoxic effect. At a cisplatin concentration of 20 μg/ml nearly no neuron survival was observed. Pre-incubation of neuronal cell cultures with recombinant human Epo for 24 h followed by co-incubation with cisplatin for another 48 h resulted in a significant protection against chemotherapeutic-induced cytotoxicity (Fig. 2). The level of cytoprotection varied from experiment to experiment. In some experiments we observed a partial, but statistically significant reduction of cisplatin-mediated neuronal death that improved neuron survival by 20-25% (Fig. 2A). In independent experiments the cytoprotection was complete with the survival of neuron cells incubated with cisplatin and Epo being higher than in untreated controls (Fig. 2B). In all experiments the cytoprotective effects of Epo were observed over the vast range of its concentration. The protective effect was observed at 0.01 U/ml and increasing the Epo concentration by up to 100-fold did not result in a better response.

Cisplatin-induced cytotoxicity against rat brain neurons results from induction of apoptosis as determined with Hoechst staining and activation of caspase-3 (27). Also in our studies cisplatin at 10 μg/ml induced a significant caspase-3 cleavage after 48 h of incubation. Epo significantly reduced the amount of active caspase-3 (Fig. 3A). It was repeatedly reported that cisplatin can induce ERK1/2 phosphorylation and that this activation is required for the cisplatin-induced apoptosis (28). Therefore, we determined the influence of cisplatin and/or Epo on ERK1/2 phosphorylation in neuron cells. We observed that after 24 h of incubation cisplatin induced a significant level of ERK1/2 phosphorylation. Interestingly, although Epo itself induced phosphorylation of ERK1/2 it strongly suppressed ERK1/2 phosphorylation in neurons co-incubated with cisplatin (Fig. 3B). Neither cisplatin nor Epo or the combination influenced phosphorylation of JNK kinase (Fig. 3C). Incubation of neurons with a selective MEK inhibitor (UO126) also protected neurons against cisplatin-induced cytotoxicity (Fig. 3D). Other inhibitors of signal transduction pathways such as LY294002 (PI-3K inhibitor), PD169316 or SB203580 (p38/MAPK inhibitors) did not exert cytoprotective effects at identical experimental conditions (Fig. 3E).

Importantly, using identical cell culture conditions we have not observed any cytoprotective effects of EPO against tumor cells of various origin including melanoma (A375 cells), breast (MDA-MB 231), ovarian (MDAH2774) or pancreatic (Mia PaCa-2) cancer (Fig. 4).

Discussion

Numerous clinical studies revealed that Epo treatment reduces transfusion requirements, increases hemoglobin concentration and improves the quality of life of cancer patients (29-31). Unexpectedly, four randomized studies revealed decreased overall survival in Epo-treated patients with advanced non-small-cell lung cancer (32) or a worse outcome of Epo treatment in patients with breast cancer receiving chemotherapy (33) and in patients with head and neck cancer receiving radiotherapy (22,34). Two other unpublished studies revealed that darbapoietin significantly shortens overall survival of patients with non-myeloid and lymphoproliferative cancers (22). The results of these studies are at odds with a recently published meta-analysis of over 40 trials with more than 8000 patients indicating that Epo treatment has no impact on overall survival of patients with cancer (35). Intriguingly, Epo has also been shown to affect the survival of cancer cells *in vitro*.

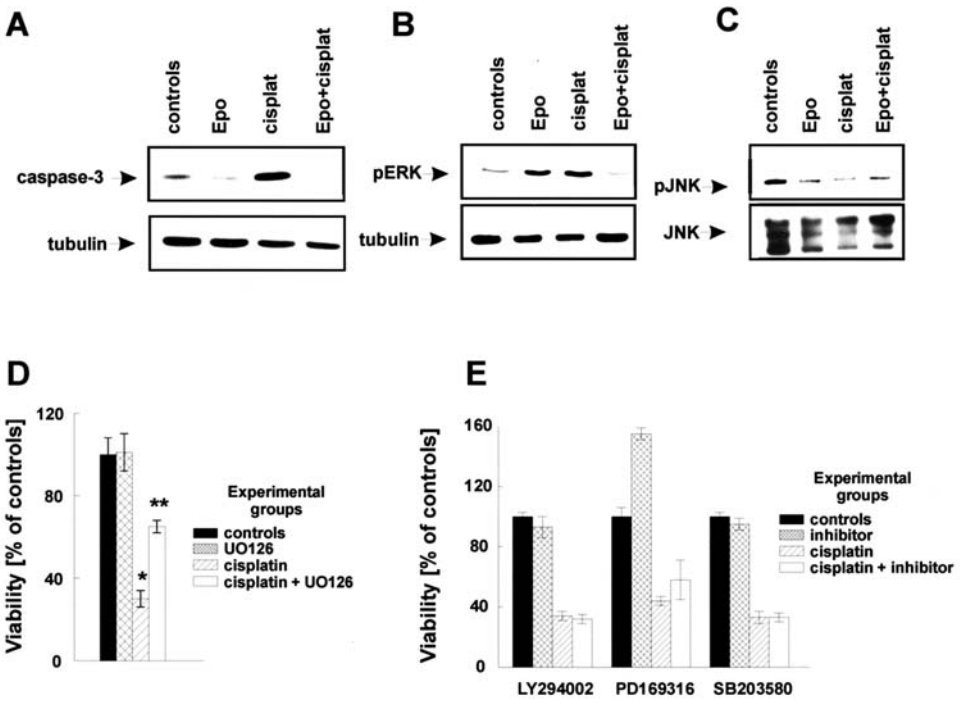

Figure 3. Epo-mediated cytoprotection results in decreased ERK1/2 phosphorylation and inhibition of cisplatin-induced caspase-3 cleavage. Freshly isolated neurons were pre-incubated with either diluents or Epo for 24 h. The incubation with cisplatin followed for another 48 h with or without fresh Epo. (A, B and C) After incubation cells were harvested and the prepared cell lysates were subjected to Western blot analysis: 50 μg of protein extracts was separated during electrophoresis. Blots were sequentially probed (after stripping) with different antibodies. (D and E) Freshly isolated neurons were pre-incubated with either diluents or indicated inhibitors for 24 h. The incubation with cisplatin followed for another 48 h with or without fresh inhibitors. The cytostatic/ cytotoxic effects were tested with MTT assay. Bars represent means ± standard deviations. *P<0.01 as compared with controls, **P<0.01 as compared with cisplatin-treated cells (Student's t-test).

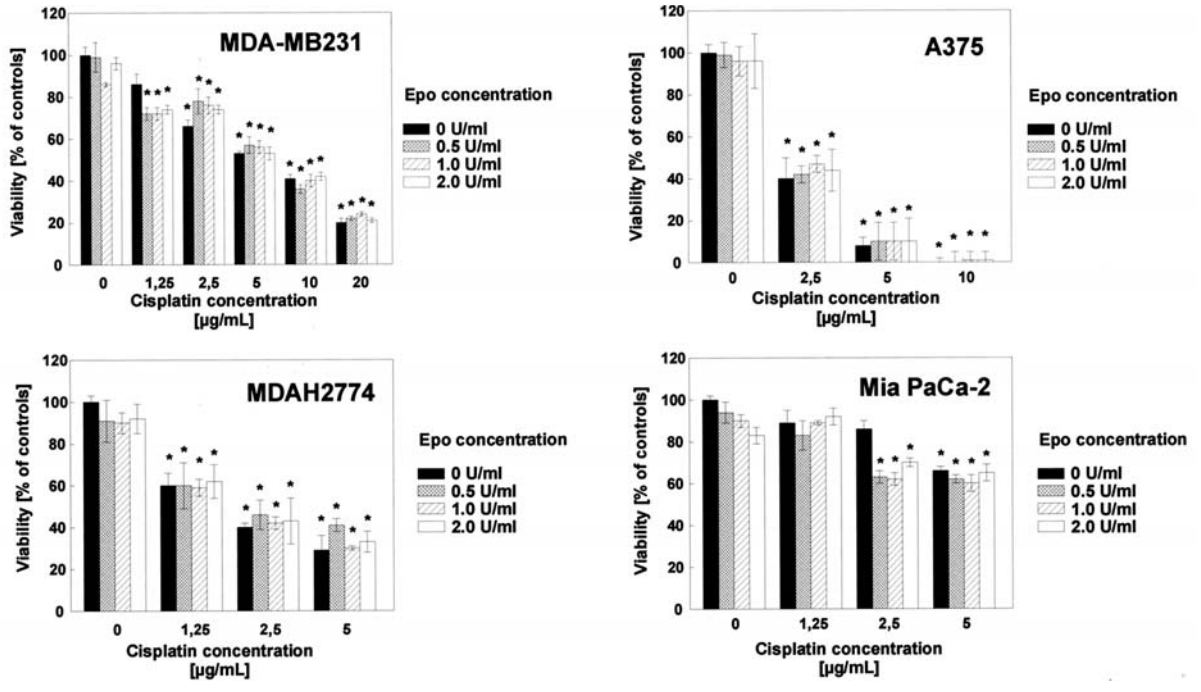

Figure 4. Epo does not protect tumor cells against cisplatin-mediated cytotoxicity. Established tumor cell lines were pre-incubated with either diluents or Epo for 24 h. The incubation with cisplatin followed for another 48 h with or without fresh Epo. The cytostatic/cytotoxic effects were tested with MTT assay. Bars represent means ± standard deviations. *P<0.01 as compared with controls (Student's t-test).

These effects were frequently reported with supraphysiological Epo concentrations and were usually small. Despite numerous reports demonstrating Epo receptors in tumor cells it must be emphasized that the studies were performed using RT-PCR or Western blotting, techniques that provide no information on the surface expression of these receptors. Antibodies used

for these studies are unsuitable for immunohistochemistry and lack specificity (36,37). Moreover, high expression of Epo receptors did not correlate with ^{125}I-labeled Epo (38). Nonetheless, we decided to use anti-EPO-R antibodies to check for the expression of Epo receptors in primary cortical neuron cultures. Despite a poor specificity of the antibodies, we have detected a prominent band of approximate molecular weight of 56-57 kDa that might correspond to EPO-R (Fig. 1).

Other researchers have also identified Epo receptors in nerve axons, in Schwann cells, in dorsal root ganglia and in cells with neuronal characteristics (39-42). The expression levels of these receptors rise under nerve injury conditions (42). Importantly, Epo can cross the blood-brain barrier (7). Therefore, it seems that Epo treatment might influence the neuron injury induced by some chemotherapeutics, such as cisplatin, which has a dose-limiting toxicity. Amelioration of cisplatin-induced neuron toxicity with Epo is of particular interest. Cisplatin-induced nephrotoxicity leads to impaired production of endogenous Epo leading to chemotherapy-associated anemia. Administration of recombinant Epo could be an effective treatment for both cancer-associated anemia and cisplatin-mediated neurotoxicity. Previous studies have demonstrated that Epo is an effective protectant against cisplatin-induced neurotoxicity (15-17). Cisplatin chemotherapy is also associated with impairment of cognitive functions that might possibly be related to the damage of brain neurons occasionally leading to encephalopathy and seizures (43,44).

We have observed various levels of Epo-mediated cytoprotection (Fig. 2) in independent experiments. In some experiments the cytoprotective effects of Epo were so strong that the survival of Epo-treated cortical neurons was superior to that in control cultures (Fig. 2B).

The ubiquitous mitogen-activated protein kinases (MAPKs) comprise a group of signaling proteins that regulate cell proliferation, differentiation, survival and adaptation responses. ERK1 and ERK2, members of the MAPK family, are important regulators of physiologic and pathologic neuronal responses. While ERK activation has beneficial cytoprotective effects in many experimental paradigms, a growing body of evidence implicates these kinases in the promotion of neuronal cell death (45-47). Similarly, inhibition of the MEK-ERK pathway has been shown to confer cisplatin resistance in tumor cells (48). Here, we have observed that Epo can directly protect brain neurons from cisplatin cytotoxicity by inhibiting excessive ERK activation and caspase-3 cleavage. The mechanism underlying this response is currently unclear especially concerning the ability of both Epo and cisplatin incubated with neurons alone to independently induce phosphorylation of ERK1/2 kinases. To further verify the role of ERK kinase activation we pre-incubated neurons with UO126, a selective ERK inhibitor. While cisplatin induced a significant cytotoxic effects towards neuron cells, UO126 pre-incubation ameliorated these effects. Other selective inhibitors of stress-associated signaling pathways (PI-3K inhibitor, JNK inhibitor, and p38/MAPK inhibitor) did not affect cisplatin-induced cytotoxicity. An identical incubation protocol with Epo pre-treatment followed by exposure to increasing concentrations of cisplatin did not ameliorate chemotherapeutic-induced cytotoxic effects towards four established human tumor cell lines.

Interestingly, Epo exerts anti-inflammatory effects that might result from reduction of primary injury that could lead to reduced production of proinflammatory cytokines, such as TNF and IL-6 (49-52). Therefore, it might be anticipated that Epo-mediated neuroprotection against cisplatin-induced toxicity might result from both direct inhibition of cytotoxicity and reduced inflammation that could be induced by the release of pro-inflammatory mediators from chemotherapeutically damaged neurons.

Altogether, our results confirm that erythropoietin is an effective neuroprotectant. Importantly, neuroprotective effects do not seem to ameliorate cisplatin-induced cytotoxicity against tumor cells of various origin. The mechanism of cytoprotection is possibly associated with impairment of excessive activation of ERK1/2 kinases and reduction of cleaved caspase-3 generation.

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