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Erythropoietin-induced cytoprotection in intestinal epithelial cells is linked to system Xc$^-$

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ABSTRACT

Necrotizing enterocolitis is a common but serious complication among premature babies. Currently, there are limited treatment options. These include intensive supportive care and surgical intervention. In this study, we hypothesize that erythropoietin (Epo) could be protective against cell necrosis by increasing the levels of glutathione. This can be regulated by increasing the activity of system xC$^-$. This was demonstrated using intestinal epithelial cells (IEC-6) as a model system. S4-CPG and sulfasalazine pharmacologically inhibit xCT, which induced cell death. Our data showed a dose dependent decrease in cell viability when treated with both inhibitors. In addition, the IEC-6 cells displayed a dose dependent increase when treated with Epo. In conclusion, Epo can be protective against cell death and ultimately be considered as a treatment option for intestinal epithelial cell death.

1. Introduction

Necrotizing enterocolitis (NEC) is the leading cause of intestinal morbidity and mortality among premature infants [26]. Although the etiology is unclear, NEC is most commonly associated with prematurity and low birth weight [23] The pathophysiology is considered to be multifactorial. An immature and underdeveloped immune system coupled with early life stress is thought to disrupt intestinal barrier function leading to bacterial translocation and intestinal injury. There are several causes of intestinal stress in the neonatal period. Emerging literature shows that anemia and blood transfusions in early life can lead to an increased frequency of NEC in premature infants [11,35]. Because of this, feeds are often held during blood transfusions in premature babies to decrease the risk of intestinal injury. The mechanism explaining the association between anemia/transfusions and NEC is not clear in the literature. Intriguingly, erythropoietin (EPO), which is given to treat chronic anemia in neonates, has recently been shown to be protective in a rodent model of NEC [25].

Erythropoietin (Epo) is well known for its' role in red blood cell production and purified in the kidney [16]. It is synthesized in the liver during perinatal and fetal period and in the kidney during adulthood. It has been used to treat various anemia of chronic disease. It is in the same protein superfamily of cytokines that include cardiotrophin 1 (CT1), ciliary neurotrophic factor (CNTF), granulocyte-macrophage ciliary-stimulating factor, interleukin 3 and 6, leukemia inhibitory factor (LIF), prolactin and thrombopoietin [28,34].

Since it's seminal discovery, Epo has gained much attention for effects on various cellular processes and mechanisms [40,41]. Several studies have demonstrated Epo as a cytoprotective protein [7,12,13,31]. The specific mechanism involved in the Epo-mediated protective effect is not fully understood. Studies support the antiapoptotic, anti-inflammatory and antioxidative mechanisms [9,13,30]. The pleiotropic effects of Epo are linked to the activation of the Epo receptor (EpoR). EpoR exists as a heteroreceptor unit consisting of a β receptor common subunit and the EpoR [3]. Mice lacking the EpoR have neuronal loss secondary to apoptosis [37]. Of note, EpoR has been found to be active in the neonatal intestine[38].

Epo has been shown to be linked to decreasing nitric oxide by inhibiting NO synthase activity in human endothelial cells [6]. Glutathione peroxidase is upregulated by Epo, which increases endogenous glutathione [15]. There is emerging data on the protective role of Epo in various cells including neural stem cells [29] and intestinal cells [10]. The mechanism in intestine has not been described but in neural stem cells it was shown that the cystine glutamate exchanger (System xC$^-$) was intimately involved.

System Xc- is an equimolar exchanger of cystine and glutamate.

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The transporter exists as a heterodimer, which includes both a regulatory (4F2hc-CD98) and catalytic subunit (xCT). 4F2hc-CD98 interacts with multiple amino acid transporters and is involved in translocation of the protein to the membrane while xCT is specific to system Xc-. The catalytic subunit consists of 12 transmembrane domains [27]. xCT activity is the rate limiting step in glutathione biosynthesis and uptakes cystine which is then reduced to cysteine. Cysteine is then joined to glutamate and glycine to form the tripeptide glutathione. Glutathione then acts as a ubiquitous regulator of reactive oxygen species. Therefore, it is plausible that inhibition of the activity of system xC^- may render cells vulnerable. In this current study, we will test the hypothesis that Epo-mediated cytoprotection in intestinal epithelial cells may require system Xc.

2. Materials and methods

2.1. IEC6 Cells

Rat intestinal epithelial cells (IEC-6) were obtained from Sigma-Aldrich. RPMI 1640 media (Mediatech Inc Manassas, VA) was used and supplemented with 8Mn glutamate, 1% penstrep, and 5% fetal calf serum. Cells were grown to 80% confluence. Cells were treated with Epo (Sigma-Aldrich Saint Louis, Missouri), hydrogen peroxide (Fisher Scientific Fair Lawn, NJ), S4-CPG (Tocris Ellisvile, MO), sulfasalazine (Sigma-Aldrich Saint Louis, Missouri) for 24 h individually. All drug treatments were done at the same time.

2.2. Cell Count

10 μL of trypan blue 0.4% solution (MP Biomedicals, LLC) was added to10 μL of each sample. 10 μL of the mixture was added to the slide. Invitrogen Countless automated cell counter was used to measure the cell viability.

2.3. Protein Concentration Assay

The Bio-Rad DC Protein Assay was used to determine the protein concentrations of the samples. The absorbencies were read on a spectrophotometer at 750 nm.

3. Immunohistochemistry for xCT

Human intestinal ileal samples from a non inflammatory condition, intestinal atresia, were stored in formalin. Samples were then embedded in paraffin following standard procedures, sectioned at 5 microm-thick sections using a rotary microtome and mounted on charged slides. For immunohistochemistry sections were deparaffinized using Citrisolv (Fisher Scientific; Waltham; MA; USA; 22–143975) and rehydrated. Antigen retrieval was achieved by immersing sections in citrate buffer at 95 °C for 20 min, allowing sections to cool down in citrate for another 20 min. Rinses between incubations were carried out in phosphate buffered-saline pH 7.4 (PBS; Sigma; Saint Louis; MO; USA; P3813). Endogenous peroxidase was quenched by incubating sections in 5% H2O2 (Sigma; 216763) in PBS for 30 min at room temperature (RT). Non-specific binding sites in the sections were blocked using 10% normal goat serum (NGS; Vector Laboratories; Burlingame; CA; USA; S1000) in PBS-0.4% Triton X100 (PBST) for one hour at RT. Sections were then incubated in a rabbit polyclonal anti-xCT primary antibody (Novus Biologicals; Littleton; CO; USA: NB300-318) diluted 1:1000 in a mixture containing 3% NGS and PBST, for 18 h at RT. The following day sections were rinsed and incubated in biotinylated goat anti-rabbit secondary antibody (Vector Laboratories; BA1000) diluted 1:400 with 3% NGS in PBST for 45 min at RT. The final step consisted in the incubation of the sections in an avidin-biotin-peroxidase complex (Vector Laboratories; PK6100) diluted 1:100 in PBS for 45 min at RT. The immunological reaction was developed using diaminobenzidine (Vector Laboratories; SK4100) under the microscope. Sections were finally rinsed and counterstained with hematoxylin using a standard protocol.

Negative controls consisted of the omission of the primary antibody and its substitution with non-immune serum. No immunoreactivity was observed in these controls.

Sections were viewed and photographed in a Zeiss Axioskop 40 microscope coupled to a Zeiss Axiocam HRc color digital camera (Zeiss; Thornwood; NY; USA). Brightness and contrast were adjusted using Corel Photopaint X7 (Corel; Ottawa; Canada). Photomontage and lettering were done using Corel Draw X7 (Corel).

4. Western-blot for xCT

Human intestinal samples collected from babies that underwent intestinal resection for a non inflammatory condition were used for western-blot. Fifteen and thirty microgram total protein were loaded onto 4–15% polyacrylamide gels (Biorad; Hercules; CA; USA; 456–8083) and separated at 150 V for 45 min. Proteins were then transferred onto polyvinylidene difluoride membranes at 100 V for one hour. Membranes were rinsed in Tris-buffered saline (TBS) and blocked for one hour in 5% non-fat dry milk (Biorad; 170–6404) in TBS containing 0.1% tween 20 (TBST) for one hour at RT. After rinsing in TBST, membranes were incubated in xCT primary antibody diluted 1:1000 in TBST 5% milk for 18 h at 4 °C. The following day membranes were rinsed in TBST and incubated in a goat anti-rabbit-horseradish peroxidase-coupled secondary antibody (Millipore; Billerica, MA; USA; AP187P) diluted 1:120000 in TBST 5% milk for one hour at RT. After subsequent rinses in TBST and TBS, membranes were incubated with ECL components (Biorad; 170–5061) and exposed using a Biorad imager running Image Lab software 5.2.1.

Membranes were reblotted for the detection of actin using mild reblot solution (Millipore; 2502) for 25 min at RT, and then blocked in TBST 5% milk for one hour at RT. After rinsing in TBST, membranes were incubated in a rabbit monoclonal anti-β-actin antibody (Cell Signaling; Danvers; MA; USA; 4970) diluted 1:1000 in TBST 5% bovine serum albumin for 18 h at 4 °C. After several rinses in TBST membranes were incubated in the same secondary antibody as for xCT, at the same dilution, and exposed as above.

Cells were triturated and pelleted then lysed with RIPA (150 mM NaCl, 50 mM Tris, 1% NP-40, 0.5% deoxycholate, 0.1% SDS, pH8.0) buffer. 15 mcg of protein was collected and ran on a SDS 12% gel. Samples were run at 120 V for 60 min. Gel was transferred to nitrocellulose using the Trans Blot Semi-Dry apparatus for 30 min at 15 V. The blot was washed three times with TBS-0.1% Tween (TBST). We blocked the membrane for 60 min with 3% milk solution. Blots were probed with xCT primary antibody (Novus Biologics) at 1:1000 dilutions in TBS-0.1% for 1 h at room temperature. A biotin anti-rabbit or anti-mouse secondary antibody (Invitrogen) along with streptavidin horseradish peroxide (Invitrogen) at a 1:1000 dilution of each in TBST for 1 h at room temperature on a rotator. Blot was developed using the Novex ECL Chemiluminescent and film with the Kodak developer.

4.1. Statistics

2 tailed Student's *t*-test and 1-way ANOVA with Dunnett multiple-comparison post-hoc test was used to analyze data with Prism 7 (GraphPad Software).

5. Results

5.1. Expression of System Xc in human intestine

The xCT system has been underreported in the intestinal epithelial cells. Fig. 1A shows that the xCT antibody recognizes a single band around 55 kDa and the actin controls for those intestinal samples.