JAK2/Y343/STAT5 signaling axis is required for erythropoietin-mediated protection against ischemic injury in primary renal tubular epithelial cells

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Erythropoietin is a potential therapy for limiting ischemic tissue injury as it has been shown in animal models to reduce ischemic injury in the brain, heart, and kidneys (5, 12, 16, 21, 22, 30). The cytoprotective properties of erythropoietin (EPO) in all tissues have been evaluated primarily in vivo and mechanisms of EPO-mediated cytoprotection, especially in the kidney, are not well-defined.

EPO signaling has been best studied in erythroid progenitor cells in the bone marrow. In erythropoiesis, ligand binding of EPO to its cognate receptor causes the autophosphorylation of Janus kinase 2 (JAK2) and subsequent phosphorylation of the eight tyrosines in the cytoplasmic domain (32). STAT5 is recruited and phosphorylated at Y343, dimerizes, and translocates to the nucleus for transcription of the B cell lymphoma Pim-3, a prosurvival STAT5 target gene, as responsive to EPO in the noninjured kidney both in vitro and in vivo. STAT5 appears to play a crucial role in cell survival under stress conditions in erythroid progenitors, we hypothesized that the JAK2/Y343/STAT5 signaling axis may also be required for EPO-mediated cytoprotection in renal tubular epithelial cells. To test this hypothesis, we used in vitro ischemic injury in primary mouse renal tubular epithelial cells and tested EPO cytoprotection using EPO receptor mutant strains with differential STAT5 signaling capabilities.

METHODS

Mice. C57Bl6 mice were purchased from Jackson Laboratory (Bar Harbor, ME). The EPO receptor mutant mice, EPOR-H and EPOR-HM, were obtained from J. Ihle, Saint Jude’s Children’s Research Hospital (Memphis, TN). EPOR-H and EPOR-HM mutants both have a truncation of the cytoplasmic domain such that all tyrosine residues except Y343, the site of STAT5 activation, have been eliminated. In EPOR-HM mice, Y343 has been mutated to phenylalanine effectively eliminating STAT5 signaling (38). Therefore, EPOR-H maintains STAT5 activation capabilities and EPOR-HM does not. EPOR-H and EPOR-HM are on a B6129PF2 genetic background. All animal protocols were approved by the Institutional Animal Care and Use Committee of Maine Medical Center Research Institute (Scarborough, ME). All mice were male and 8–16 wk of age at the time of testing.

Cell culture of primary renal tubular epithelial cells. Mouse kidneys were collected, decapsulated, and the medullas were dissected and discarded. For each kidney, the cortices were minced and digested with collagenase type 4 (Worthington Biochemical, Lakewood, NJ) at 200 U/ml and soybean trypsin inhibitor (SBTI; Invitrogen, Carlsbad, CA) suspended in Hanks balanced salt solution (HBSS; Invitrogen). Cells were digested for 30 min at 37°C with rotation at 70 rpm. Following enzyme inactivation and density sedimentation using serum, cells were resuspended in kidney culture media (KCM) consisting of DMEM/F-12 (Invitrogen) base media and insulin 5 μg/ml/transferrin 2.75 μg/ml/selenium 3.35 ng/ml (Invitrogen), 2.0 μg/ml APO transferrin (Sigma, St. Louis, MO), 40 ng/ml hydrocortisone (Sigma), 0.1 μg/ml recombinant human epidermal growth factor (rhEGF from R&D Systems, Minneapolis, MN), and 1% antibiotic/antimycotic solution (Sigma, 10,000 U/ml penicillin, 0.1 mg/ml streptomycin, and 0.25 μg/ml amphotericin B). Cells were plated on Nunclon-treated tissue culture plates in 96-well plates for the lactate dehydrogenase (LDH) assay or 6-well plates for Western blot and quantitative RT-PCR experiments. Cell cultures were incubated at 37°C with 5% CO2. Culture media were replaced initially at 24 h and subsequently every 48–72 h using KCM without rhEGF. Tests to rule out the presence of mycoplasma were not performed.

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Ischemic injury induction. Ischemic injury was induced on days 7–10 when renal tubular epithelial cell (RTEC) cultures had grown to 80–90% confluency using methods we recently described (4a).

Hypoxia. A hypoxic (1% oxygen) environment was achieved using the Modular Incubator Chamber (Billups-Rothenberg, Del Mar, CA). To do this, each chamber was purged for 4 min with a certified gas mixture consisting of 5% CO2–94% nitrogen. Chambers were incubated at 37°C for 30 min, repurged for an additional 4 min, and incubated at 37°C for 12, 18, 24, or 48 h. The cell culture media for chamber hypoxia experiments were DMEM without dextrose.

Chemical anoxia. ATP was depleted using antimycin A and 2-deoxy-D-glucose (Sigma). Two micromolar antimycin A was prepared in DMEM without dextrose (Invitrogen) from a stock solution of 200 μM antimycin A in ETOH. Four millimolar 2-deoxy-D-glucose was prepared in DMEM without dextrose. Cultures were incubated at 37°C with 5% CO2 before injury induction. Alternatively, EPO was added to cell cultures at the time of ischemia induction (no preincubation). To ensure that any cytotoxic effects were due to EPO alone, a stock concentration of EPO vehicle was tested under all injury conditions and at each time point.

In vitro EPO response. Cells were starved in DMEM without dextrose for 24 h, media were aspirated, and either EPO or vehicle was added at 50 U/ml in DMEM without dextrose for 15, 30, or 120 min for Western blot analysis. Protein bands were detected by enhanced chemiluminescence (Amersham Biosciences) and visualized using the X-OMAT 2000A processor (Eastman Kodak, Rochester, NY).

In vivo EPO response. C57Bl/6 mice were anesthetized with isoflurane. EPO (Procrit, Amgen) was administered via tail vein injection. Control mice were injected with a weight-specific volume of 0.9% NaCl. After 30 min, kidneys were perfused with 1× PBS (Invitrogen) and surgically removed. Tissue was flash-frozen in liquid nitrogen and stored at −70°C. Tissue was homogenized using a proprietary TR-50 lysis solution (Autogen, Holliston, MA) and the AutoDisrupter 24 tissue homogenizer (Autogen).

Quantitative RT-PCR. Total RNA was extracted from cultured cells and tissue using the AutoGenPrep 245 (AutoGen). Cultured cells were collected in TRIzol reagent (Invitrogen) and preextracted with chloroform. Following extraction, RNA was treated with rDNASE I (Ambion, Austin, TX) according to the manufacturer’s protocol and RNA integrity was visualized on a 1% denaturing gel using ethidium bromide. cDNA was prepared using iScript (Bio-Rad, Hercules, CA). Primers (Integrated DNA Technologies, Coralville, IA) were designed using Beacon designer software (Bio-Rad). Optimal annealing temperatures and the efficiency of each primer set were determined before use. Quantitative RT-PCR was performed using iQSupermix SYBR Green I (Bio-Rad) and amplified on the iCycler thermocycler. Thermal cycling conditions were 95°C for 3 min, 95°C for 0.3 min, followed by 50 cycles at 54–59°C depending on the primer pair being used, and 72°C for 0.3 min. The relative mRNA expression of each gene was normalized to β-actin. The Pfaff formula was used for calculations of relative mRNA expression (23). Each cell culture experiment was performed a minimum of three times. Each PCR reaction was done in triplicate wells. A fold change in gene expression greater or less than 1.5 was considered significant.

Primer sequences. β-actin forward: 5’-CGT GGC TGA CAT TAA AGA GAA G-3’; β-actin reverse: 5’-TGG ATG AGA CCA CAG GAT TCCATA-3’; Pim-1 forward: 5’-GGT GCT CAA GGA GAC AGT CTA GAC-3’; Pim-1 reverse: 5’-TGC CGT GGT AGC GAT GGT AGC-3’; Pim-2 forward: 5’-AGT GTA TAG CCC TCC TGA GTG-3’; Pim-2 reverse: 5’-CAC AGA CCA TGT CAT AGA GTA GG-3’; Pim-3 forward: 5’-ATG CTT CGT TCA AAC TGC-3’; Pim-3 reverse: 5’-ACC TGG TAC ACC TTC TCG-3’; Bcl-xL forward: 5’-CTT TGA GCA GGT AGA GAA TAC-3’; Bcl-xL reverse: 5’-GTC CGT GGT AGC GAT GGT AGC-3’; Bcl-2 forward: 5’-AGT GTA TAG CCC TCC TGA GTG-3’; Bcl-2 reverse: 5’-CAC AGA CCA TGT CAT AGA GTA GG-3’; C-erbB-2 forward: 5’-ATG CTT CGT TCA AAC TGC-3’; C-erbB-2 reverse: 5’-ACC TGG TAC ACC TTC TCG-3’; Bcl-xL forward: 5’-CTT TGA GCA GGT AGA GAA TAC-3’; Bcl-xL reverse: 5’-GTC CGT GGT AGC GAT GGT AGC-3’; C-erbB-2 forward: 5’-ACC TGG TAC ACC TTC TCG-3’; C-erbB-2 reverse: 5’-GTC CGT GGT AGC GAT GGT AGC-3’; C-erbB-2 reverse: 5’-ATG CTT CGT TCA AAC TGC-3’.

Statistical analysis. All values are expressed as means ± SE. The mean LDH release in test (with EPO) vs. control (no EPO) groups was
RESULTS

EPO cytoprotection in the setting of necrotic ischemic injury. The ability of EPO to be cytoprotective under previously established necrotic ischemic injury conditions was tested. Necrotic injury was measured by the amount of LDH released into culture supernatants above baseline LDH release. Mild injury was defined as ≤20% LDH release, moderate injury as 20–49% LDH release, and severe injury as ≥50% LDH release. Preincubation with EPO for 4 and 6 h but not 2 h significantly reduced the LDH release when injury was induced by antimycin A (Fig. 1A). A 6-h preincubation with EPO was necessary to reduce injury induced by chamber hypoxia and 2-deoxy-D-glucose (Fig. 1, B–C). LDH release was not reduced by EPO if EPO was introduced at the time of injury (no preincubation) induced by antimycin A or chamber hypoxia (Fig. 2, A–B) but significantly reduced by EPO from injury induced by 2-deoxy-D-glucose (Fig. 2C). Preincubation with EPO for 6 h protected against injury induced by antimycin A, chamber hypoxia, and 2-deoxy-D-glucose in mild-moderate injury but not severe injury (Fig. 2, D–F).

EPO cytoprotection against necrotic ischemic injury is JAK2 dependent. JAK2 phosphorylation following EPO stimulation has been demonstrated in the human kidney 2 (HK-2) immortalized cell line and in human but not mouse primary RTEC (26, 30). As verification of EPO signaling in primary murine RTEC, phosphorylation of JAK2 in response to EPO was tested. As determined by Western blotting (Fig. 3A), JAK2 but not JAK1 was phosphorylated by addition of EPO to RTEC at 5, 15, and 30 min (JAK2) or 15, 30, and 120 min (JAK1). To determine whether EPO cytoprotection was JAK2 dependent, AG490, a JAK2 inhibitor, was utilized (34, 36). EPO reduced injury induced by antimycin A when preincubated with KCM or with DMSO, the AG490 vehicle control. Preincubation with EPO for 6 h in the presence of AG490, however, eliminated EPO cytoprotection (Fig. 3B).

JAK2/Y343/STAT5 signaling axis is required for EPO cytoprotection in necrotic ischemic injury. Although JAK2 phosphorylation and activation of downstream survival pathways have been demonstrated in ischemic injury to RTEC, the requirement for STAT5 activation in EPO cytoprotection has not been determined (26). RTEC from EPOR-H and EPOR-HM mice were cultured and subjected to the same injury conditions as wild-type (WT) mice. There was no significant difference in the LDH release between RTEC from WT, EPOR-H, or EPOR-HM mice when ATP was depleted using antimycin A or with chamber hypoxia (Fig. 4B). 2-Deoxy-D-glucose produced more injury in RTEC from EPOR-HM (38% LDH release) compared with WT (26%); however, this was within the range of injury (10–50% LDH release) at which EPO was shown to be cytoprotective. Preincubation with 50 U/ml EPO for 6 h before injury induced by antimycin A for 12 h reduced the LDH release in WT and EPOR-H but not EPOR-HM mice (Fig. 5A). In separate experiments, an 18-h exposure to 2 μM antimycin A produced identical results (n = 4, data not shown). EPO also reduced the LDH release in WT and EPOR-H but not EPOR-HM mice in injury induced by chamber hypoxia for 24 h (Fig. 5B) and 2-deoxy-D-glucose for 48 h (Fig. 5C).

Pim-3 is an EPO-responsive gene in the kidney in vitro and in vivo. Gene transcription in response to EPO was tested by quantitative RT-PCR. Cultures of primary RTEC from WT mice were starved for 24 h in DMEM F-12 and exposed to 100 U/ml EPO or vehicle media without EPO (DMEM F-12) for 0.5, 1, 2, 4, and 6 h. As shown in Fig. 6A, Bcl2, Bcl-xL, and Cis gene expression was not affected by EPO. Pim-1 transcripts increased in response to EPO at 0.5, 1, and 2 h, gradually declining at 4 and 6 h to levels seen with no EPO stimulation. Pim-2 transcripts were unaffected by EPO, whereas Pim-3 transcripts were significantly upregulated by exposure to EPO for 0.5 and 1 h with mRNA levels gradually decreasing to baseline levels at 4 and 6 h (Fig. 6B).

For in vivo experiments, WT mice received intravenous injections of EPO at 5,000 U/kg 30 min before perfusion and collection of kidneys. Pim-1, although upregulated in response to EPO in vitro, failed to respond to EPO stimulation in vivo.
In support of the in vitro results, Pim-2 was nonresponsive to EPO stimulation in vivo while Pim-3 transcripts were significantly increased in response to EPO (Fig. 6C).

DISCUSSION

In this study, we demonstrate for the first time that EPO protects against necrotic ischemic injury in primary mouse RTEC via the JAK2/Y343/STAT5 signaling axis. We also identify Pim-3 as an EPO-responsive gene in the kidney under noninjury conditions. The response of Pim-3 to EPO during ischemic injury has not been tested and is an area for future investigations.

Ischemic injury to renal tubular epithelia is an important mechanism in the development of tubular injury which may result in apoptosis or necrosis depending on the severity and duration of the ischemic insult (3, 13). We previously examined conditions for producing reproducible necrotic ischemic injury in primary mouse RTEC. Using necrotic ischemic injury induction to study EPO cytoprotection, we demonstrated that EPO protects against mild-moderate ischemic injury (e.g., 10–50% LDH release) but not against severe ischemic injury (>50% LDH release). Previously, ATP levels have been measured in mouse primary RTEC and in the immortalized porcine proximal tubule cell line (LLC-PK) by luciferase activity and by HPLC following injury induction by antimycin A and 2-deoxy-D-glucose (8, 27). The measurement of ATP in these studies indicated whether cells died by apoptosis or necrosis. Our study demonstrated the ability of EPO to reduce mild-to-moderate but not severe necrotic injury as indicated by LDH release. Although LDH released from the cytosol is considered a standard measure of necrotic injury, it is likely that there is overlap in apoptotic and necrotic cell death processes. LDH release is therefore not completely specific for necrotic cell death and to what extent cells may have died by apoptosis cannot be determined by measurement of LDH release.

The binding of EPO to the EPO receptor results in the autophosphorylation of JAK2. Our data show that JAK2 was phosphorylated in response to EPO and addition of AG490, a JAK2 inhibitor, eliminated EPO cytoprotection. AG490 has been used extensively as a JAK2 inhibitor both in vitro and in vivo in a variety of cells and tissues (10, 14, 20, 24, 33, 36). However, AG490 is not totally specific for JAK2 inhibition as it has also been shown to inhibit the autophosphorylation of epidermal growth factor receptor kinase, constitutive activation of STAT3 DNA binding, and IL-2-induced growth of MF tumor cells, as well as the autokinase activity of JAK3 in T lymphocytes (15, 31). The inability of AG490 to inhibit JAK3 in colorectal cancer cells as reported by Xiong et al. (33) indicates the specificity of AG490 may be cell and tissue context dependent. In vivo administration of AG490 is well-tolerated in mice indicating that it is not a general kinase inhibitor. In support of this, lymphocytic tyrosine kinases shown not to be inhibited by AG490 include Lck, Lyn, Btk, Syk, Zap70, and p56Lck (14, 19). The addition of AG490 offers a viable method to demonstrate JAK2 inhibition in primary mouse RTEC which may not be amenable to other methods such as viral transfection or siRNA. AG490, which continues to be extensively used as a JAK2 inhibitor both in vitro and in vivo, was used in this study as it remains as specific a JAK2 inhibitor as is currently available for demonstration of inhibition of JAK2 phosphorylation and activation in nonimmortalized primary RTEC.

EPO-mediated protection from injury in the kidney has been attributed to activation of PI3K/AKT, and downstream events including inhibition of caspase activity, upregulation of anti-apoptotic members of the Bcl2 family such as Bcl-xL, and inactivation of the proapoptotic protein BAD (7, 26, 28). The contribution of STAT transcription factors to EPO cytoprotection in tissues outside the erythroid compartment is uncertain.
Utilizing EPO receptor mutants with positive (EPOR-H) or negative (EPOR-HM) STAT5 signaling capabilities, we demonstrated that EPO significantly reduced ischemic injury to RTEC from WT and EPOR-H but not EPOR-HM mice. These results indicate EPO protection in the setting of ischemia-induced necrosis occurs via the JAK2/Y343/STAT5 signaling axis and therefore STAT5 may be a crucial mediator for EPO-induced cell survival. These results are analogous to those found in the bone marrow, where erythropoiesis under stress conditions has been shown to require the JAK2/Y343/STAT5 signaling axis (18). In the nervous system, there are conflicting data regarding the role of STAT proteins in EPO cytoprotection. In primary cortical neurons, EPO reduced ischemic injury due to oxygen glucose deprivation by activation of PI3K/AKT but failed to induce STAT1, STAT3, STAT5, or the STAT5 targets Bcl2 and Bcl-xL (24). Digicaylioglu and Lipton (9) demonstrated that EPO protection against ischemic and excitotoxic injury to primary cortical neurons was due to JAK2-dependent activation of NF-\(\kappa\)B. STAT proteins were not investigated in this study. EPO activation of STAT5 in response to hypoxia is likely to be tissue specific and influenced by the nature and severity of injury.

Fig. 3. Inhibition of JAK2 by AG490 blocks EPO cytoprotection. Top: confluent cultures of RTEC from WT mice were starved for 24 h in DMEM F-12 media with no additives. Kidney culture media (KCM) alone or with EPO at 50 U/ml was added for 5, 15, and 30 min for JAK2 and 15, 30, and 120 min for JAK1. EPO caused the phosphorylation of JAK2 but not JAK1 at each time point. Bottom: before injury induction, confluent cultures of RTEC from WT mice were preincubated with AG490, DMSO, KCM (no EPO), 50 U/ml EPO, 50 U/ml AG490 + EPO, or 50 U/ml DMSO + EPO prepared in KCM. Media were removed and injury was induced by incubation with antimycin A for 12 h. EPO preincubation resulted in a 35% reduction in LDH release when present alone (lane 4, \(P = 0.035\)) or in the presence of DMSO, the vehicle control for AG490 (lane 6, \(P = 0.046\)). Preincubation with AG490, a JAK2 inhibitor (lane 5), completely interfered with EPO cytoprotection (\(n = 3\)).

Short intermittent exposure to hypoxia in vivo has been shown to reduce injury to subsequent ischemic episodes, a phenomenon known as ischemic preconditioning which has been demonstrated in the heart, kidney, and brain (2, 4, 5, 25). In studies performed in vitro and in vivo in the heart, the activation of STAT5 by either Src kinase or JAK has been shown to be essential for the cardioprotective effects of preconditioning (34). Src kinase activation of STAT5 appears to mediate preconditioning through the PI3K/AKT survival pathway; however, the preconditioning pathway involved in JAK activation of STAT5 is unknown (34). The beneficial effects of ischemic preconditioning in the kidney have been attributable to decreased p38 and JNK activation with no effect on ERK1/2. The role of STAT proteins has not been investigated (4). Our results show that preconditioning with EPO was necessary for EPO-mediated protection from ischemic injury with extended preconditioning times (6 h) producing the most significant reduction in LDH release. Preincubation failed to protect EPOR-HM tubular epithelial cells from injury. These results indicate that the STAT5 pathway mediates EPO cytoprotection and suggest that STAT5 may also be important in ischemic preconditioning in the kidney.

Fig. 4. Ischemic injury in EPOR-H and EPOR-HM. A: 2 strains of mice with mutations of the EPO receptor were used to define the contribution of the JAK2/Y343/STAT5 signaling pathway to EPO cytoprotection. EPOR-H contains a truncation of the distal half of the cytoplasmic domain with all tyrosines eliminated except Y343 which is the site of STAT5 activation. EPOR-HM contains the same truncation as well as a mutation of the residual tyrosine, Y343, to phenylalanine which blocks STAT5 activation. B: RTEC from EPOR-H and EPOR-HM mice were cultured and ATP depleted using antimycin A, chamber hypoxia, and 2-deoxy-D-glucose. The LDH release due to ischemia was comparable for all 3 strains (\(n = 4\)).
The Pim genes (Pim-1, -2, and -3) are serine/threonine kinases which inhibit apoptosis and regulate cellular metabolism (1). We hypothesized that Pim may also be an EPO-responsive STAT5 target gene in the kidney. Our results show a significant increase in gene transcription of Pim-3 in response to EPO in the kidney both in vitro and in vivo. The responses of Pim genes to EPO administration have been examined under noninjury conditions at the transcript level only and have provided initial evidence that Pim-3 may be important to the mechanism underlying EPO cytoprotection. Conclusions about the role of Pim genes in EPO cytoprotection cannot be made with the data presented. Future experiments to test protein levels of Pim as well as the ability of
EPO to reduce injury in Pim knockout mice will provide insight into the importance of the Pim genes to EPO cytoprotection in the kidney. The use of EPO receptor knockout mice, EPOR-H and EPOR-HM, in future experiments will also clarify the dependency of Pim-3 activation on STAT5 signaling in response to EPO.

In summary, we demonstrate for the first time that the JAK2/Y343/STAT5 axis is a crucial mediator of EPO-mediated protection against ischemic injury in RTEC. Further studies will need to be conducted to define the role of specific STAT5 target genes in EPO cytoprotection. As EPO administration has been shown to mimic the effects of ischemic preconditioning, the role of STAT5 in ischemic preconditioning in the kidney also warrants further investigation.

REFERENCES