Reviewer's report

Title: No evidence for protective erythropoietin alpha signalling in rat hepatocytes

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Reviewer: Constance Noguchi

Reviewer's report:

In light of reports of protection from ischemia-reperfusion injury in rodents by erythropoietin (EPO), the authors examine the potential for EPO to directly protect hepatocytes from hypoxia-reoxygenation and cold-induced injury. Expression of EPO receptor (EPOR) mRNA is detected by PCR in cultures of hepatocytes isolated from rat livers. Incubation with EPO under select conditions reduced cell injury by hypoxia as determined by lactate dehydrogenase (LDH) release, but this was also observed with EPO treated with heat-inactivation (attributed to glycine in EPO treatment). No EPO induction of Bcl-2 and EPO specific reduction in LDH release by cold is observed, and activation of JAK2 and STAT5 in EPO perfused rat livers was not detected. Therefore, the authors conclude that there is no evidence for a direct EPO protective effect on rat hepatocytes.

Critique:

Major Compulsory Revisions:

The EPO protective activity for hepatic ischemia-reperfusion injury reported in several animal models suggest EPOR expression and function beyond hematopoietic tissues. Of particular interest is the mechanism of EPO protection via direct or indirect activity in non-hematopoietic tissue that express low level of EPOR compared with erythroid progenitor cells. The evidence for EPOR mRNA expression in hepatocytes presented here are incomplete. In addition, Pinto et al. (Blood 2008) reported that EPO mediated regulation of hepcidein expression via EPOR signaling and C/EBPalpha expression in primary mouse hepatocytes. Although they observed no statistical differences in viability with EPO treatment, their work provides evidence for functional EPOR production in primary hepatocytes and should be included in the discussion. The data presented here extends the culture conditions to hypoxia-reoxygenation and cold-induced injury. A positive control (glycine) is included for hypoxic injury and was useful in explaining the protection observed with heat-inactivated EPO, although it is unclear why protection is greater with EPO heat-inactivation. However, no positive control is provided for cold-induced injury. Controls for pJAK2 and pSTAT5 are also lacking. The authors should discuss the possible indirect mechanisms that may account for EPO protective activity in liver.

Specifics:

1. Figure 5: Data on EPOR expression in hepatocytes is incomplete. The PCR in
(A) only shows a portion of the EPOR transcript. Is the mature erythroid form of the EPOR identical to the transcript produced in hepatocytes? Has this been shown previously? How does the level of expression indicated in (B) compare with levels produced in an erythroid control such as erythroid progenitor cells, bone marrow or UT-7 cells?

2. Figure 1: Why does heat-inactivation increase the protective effect of EPO observed in Figure 1?

3. A positive control for protection to cold-induced injury should be included.

4. Figure 7: What are the bands close to pJAK2 and pSTAT5 in the perfused rat liver extracts? Which are the correct pJAK2 and pSTAT5 bands? The comparison with total JAK2 and STAT5 should be provided, unless these Western blots are intended to show complete absence of pJAK2 and pSTAT5. If this is the case, then some treatment to show that activation of JAK2 and STAT5 can be detected by some other treatment should be included.

5. If EPO does not stimulate hepatocytes directly, what are the other possible mechanisms for the reported protection from hypoxia-reoxygenation in the liver?

Minor Revisions:
1. Figure 5: What was the time in culture prior to analysis of the hepatocytes used in this study?

Discretionary Revisions:
1. Figure 1: The protein content of recombinant human EPO sold commercially consists primarily of albumin as carrier. Therefore, if the protection shown in figure 1 is non-specific and related to glycine, is it also present with addition of albumin alone in an analogous buffer similarly treated?

2. Figure 5: Pinto et al., (Blood 2008) examined primary mouse hepatocytes immediately following isolation. Does EPOR increase, decrease or remain unchanged when analyzed immediately following isolation? What is the level of EPOR in the perfused liver?

3. Figure 3: The protection by glycine should also be included here for the analysis of nuclear morphology.

4. Figure 6: Josefsen et al. (Stem Cells 2000) showed expression of Bcl-xL in CD34(+) hematopoietic progenitor cell cultures at 4 days of EPO treatment which then increased with culture time. Does longer EPO treatment of hepatocytes beyond 12 hours affect the results presented here?

5. Figure 7. The results for cultured hepatocytes should be shown.

Level of interest: An article whose findings are important to those with closely related research interests
Quality of written English: Acceptable

Statistical review: No, the manuscript does not need to be seen by a statistician.

Declaration of competing interests:
I declare that I have no competing interests.