Erythropoietin mediates tissue protection through an erythropoietin and common β-subunit heteroreceptor

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Contributed by Anthony Cerami, September 2, 2004

The cytokine erythropoietin (Epo) is tissue-protective in preclinical models of ischemic, traumatic, toxic, and inflammatory injuries. We have recently characterized Epo derivatives that do not bind to the Epo receptor (EpoR) yet are tissue-protective. For example, carbamylated Epo (CEpo) does not stimulate erythropoiesis, yet it prevents tissue injury in a wide variety of *in vivo* and *in vitro* models. These observations suggest that another receptor is responsible for the tissue-protective actions of Epo. Notably, prior investigation suggests that EpoR physically interacts with the common β receptor (βcR), the signal-transducing subunit shared by the granulocyte-macrophage colony stimulating factor, and the IL-3 and IL-5 receptors. However, because βcR knockout mice exhibit normal erythrocyte maturation, βcR is not required for erythropoiesis. We hypothesized that βcR in combination with the EpoR expressed by nonhematopoietic cells constitutes a tissue-protective receptor. In support of this hypothesis, membrane proteins prepared from rat brain, heart, liver, or kidney were greatly enriched in EpoR after passage over either Epo or CEpo columns but covalently bound in a complex with βcR. Further, antibodies against EpoR coimmunoprecipitated βcR from membranes prepared from neuronal-like P-19 cells that respond to Epo-induced tissue protection. Immunocytochemical studies of spinal cord neurons and cardiomyocytes protected by Epo demonstrated cellular colocalization of Epo βcR and EpoR. Finally, as predicted by the hypothesis, neither Epo nor CEpo was active in cardiomyocyte or spinal cord injury models performed in the βcR knockout mouse. These data support the concept that EpoR and βcR comprise a tissue-protective heteroreceptor.

Erythropoietin (Epo) is a cytokine characterized by remarkable tissue-protective activity in preclinical models of neuronal, retinal, cardiac, and renal ischemic injury (reviewed by Grasso *et al.* in ref. 1). A recent positive clinical study showing that administration of recombinant human Epo (rhEpo) benefits stroke patients (2) provides hope that additional translation from preclinical models of tissue protection into other human diseases will occur. The broad efficacy of Epo observed in model systems depends on Epo's key role in multiple protective pathways activated in many diseases, including an inhibition of apoptosis, restoration of vascular autoregulation, attenuation of inflammatory responses, and augmentation of restorative functions, including the direct recruitment of stem cells (1).

The signaling pathways in these responses have not been fully clarified but are known to involve multiple second messenger systems (reviewed by Ghezzi and Brines in ref. 3). Notably, the results of previous studies have shown that the affinity of Epo for the neuronal-type receptor is substantially lower than that of Epo for the red-cell precursor receptor homodimer $(EpoR)_2$ (4). Further, neuronal proteins associated with Epo in cross-linking studies are smaller than those isolated from bone marrow (5). Finally, our recent work identifying Epo derivatives that lack hematopoietic activity yet retain full tissue protection confirm the distinct nature of the tissue-protective Epo receptor (EpoR) (6).

Several laboratories have previously reported a physical association (7) and functional interaction (8) of EpoR with the common β receptor (βcR) subunit, also known as CD131, which provides increased ligand-binding affinity to the receptor complex and is also the signal-transducing component common to the granulocyte-macrophage colony stimulating factor (GM-CSF), IL-3, and IL-5 receptors (reviewed in ref. 9). However, the significance of these observations was questioned when it was shown that βcR knockout mice exhibit normal hematopoiesis (10).

In light of the successful separation of Epo hematological and tissue-protective activities, we reassessed the relationship between EpoR and βcR in tissues exhibiting Epo protection as a potential explanation of how a nonerythropoietic Epo initiates tissue protection. A number of studies involving cells within the CNS, including microglia, have reported expression of βcR and responsiveness to IL-3, IL-5, and GM-CSF *in vivo* and *in vitro* (11), indicating that βcR is functionally present in brain cells. Other tissues, however, have not been examined for coexpression of EpoR and βcR. In the present communication, we demonstrate that EpoR and βcR are coexpressed in Epo-sensitive cells within protected tissues. Moreover, EpoR and βcR copurify on a variety of affinity resins and in immunoprecipitation experiments. Finally, Epo is not tissue-protective in the βcR knockout mouse. Together, these results are consistent with a model wherein tissue protection is mediated through a heteroreceptor complex comprising both EpoR and βcR.

Methods

Animals. All protocols were approved by the Animal Use and Care Committee of the Kenneth S. Warren Institute in accordance with the directives of the Guide for the Care and Use of Laboratory Animals. The βcR knockout mice used for these experiments are described in ref. 10, and we thank L. Robb (Royal Melbourne Hospital, Victoria, Australia), C. G. Begley (Amgen), J. A. Whitsett (Children's Hospital Center, Cincinnati), and W. Hull (Children's Hopsital Center, Cincinnati) for providing this strain. Confirmation of βcR−/− was accomplished for each mouse by PCR genotyping with primers described in ref. 10. Control strain-matched, wild-type mice (C57/BL6) and Sprague–Dawley rats were obtained from Taconic Farms.

Abbreviations: βcR, common β receptor; Epo, erythropoietin; CEpo, carbamylated Epo; EpoR, Epo receptor; GM-CSF, granulocyte-macrophage colony stimulating factor; rhEpo, recombinant human Epo.

†M.B., P.G., Q.X., C.S.-R., E.P., D.D., D.G., C.H., T.C., and A.C. are minority stockholders of Warren Pharmaceuticals, which is engaged in commercializing tissue-protective cytokines.

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Materials. All reagents not specified were of the highest purity and obtained from local suppliers. Carbamylated Epo (CEpo) was prepared as described in ref. 6 and confirmed to be nonerythropoietic at concentrations up to 10 μg/ml by using TF-1 and UT-7 cells. Epo was a generous gift from Dragon Pharmaceuticals (Vancouver).

Immunocytochemistry. Animals were perfused with 4% paraformaldehyde, and tissues were removed, embedded in paraffin, cut into 6-μm-thick sections, and processed as described in ref. 12. Antibodies used (βcR: K-17, N-20; EpoR: M-20, H194) were obtained from Santa Cruz Biotechnology.

Affinity Purification of Cell Membranes. Epo and CEpo columns were prepared by adding 3.5 mg of recombinant protein to cyanogen bromide-activated Sepharose 4B in a conical tube and slowly rotating it at 4°C for 48 h. Efficiency of coupling ($\approx$100%) was determined by UV spectrometry of the supernatant. Membranes obtained from freshly dissected organs from normal rats were minced and homogenized in phosphate buffer with a protease inhibitor mixture of phenylmethylsulfonylfluoride (1 mM) and aprotinin (10 μg/ml). After centrifugation (30 min at 15,000 $\times$ *g* in microfuge tubes), the supernatant was passed over a lentil lectin Sepharose 4B column (Amersham Biosciences). Retained glycoproteins were eluted by α-methylmannose (10 μM) and subsequently analyzed by Western blotting or affinity purification over Epo or CEpo columns.

EpoR Immunoprecipitation. P19 cells were grown to 70% confluence as described in ref. 13, treated with 10 ng/ml Epo or saline for 15 min, and detached by gently swirling the flask. The cells were collected by centrifugation (7 min at 1,000 $\times$ *g*) and resuspended in lysis buffer [Tris-buffered saline with protease inhibitors phenylmethylsulfonylfluoride (1 mM) and aprotinin (10 μg/ml), 2 mM $CaCl_2$, 1% Triton, and 1% Nonidet P-40]. Freezing and vortexing were avoided. After removal of cellular debris by centrifugation for 10 min (1,000 $\times$ *g*), the lysate was diluted to a final concentration of $\approx$1 mg of protein per ml and was incubated with protein A Sepharose (10 μl of drained gel per ml; Amersham Biosciences) for 1 h at room temperature to reduce nonspecific binding. The supernatant was then incubated with protein A Sepharose (10 μl of gel per ml) that was previously coupled to the antibody for 1 h and then washed three times with lysis buffer. Either an antibody against the common β chain (K17, Santa Cruz Biotechnology) or a mixture of two antibodies against EpoR (M20 and H194, Santa Cruz Biotechnology) at a final dilution of 1:200 was used for an overnight incubation at 4°C. The protein A Sepharose beads, either alone (nonspecific) or antibody-coupled (specific), were then washed five times with low-detergent lysis buffer (the same as above but containing 0.5% Triton without Nonidet P-40), and bound proteins were dissociated by the addition of 30 μl of 2$\times$ Laemmli sample buffer with 5% 2-mercaptoethanol and run on a 10% SDS/PAGE. Immunoblotting was performed with either the antibody against βcR (K17, Santa Cruz Biotechnology) or an antibody against EpoR (H194, Santa Cruz Biotechnology) at 1:200 and with 1:50,000 anti-rabbit horseradish peroxidase secondary antibody (Sigma) and was detected by the ECL Plus system (Amersham Biosciences).

Spinal Cord Injury. Spinal cord compression in mice was performed by using a slight modification of the protocol of Farooque (14) under isoflurane anesthesia and a controlled core temperature of 35–37°C. Briefly, C57/BL6 wild-type or βcR knockout mice (15) of 8–16 weeks of age (10 animals per group) were subjected to a T3 laminectomy, and a 2-mm stainless steel rod (15 g) was then applied to the dura with a micromanipulator for 4 min. A single dose of CEpo or Epo (10 μg/kg) was administered i.p. immediately after injury. During recovery, animals were assessed in a blinded fashion by using the scoring system of Basso *et al.* (16) and of Tarlov (17). The bladders were manually expressed twice daily for each animal until neurogenic function developed, usually by 10 days.

Isolated Ventricular Cardiomyocytes in Primary Culture. Left ventricular cardiomyocytes were isolated from adult wild-type C57/BL6 or βcR knockout mice (15) as described in ref. 18. Briefly, hearts were perfused via the aorta with collagenase buffer (type II, Worthington) gassed with 85% O_2 and 15% N_2 at 37°C. Left ventricular myocytes were then isolated by mechanical dissociation, separated by differential centrifugation, plated on laminin-coated dishes, and maintained in minimum essential medium with Hanks' salts and L-glutamine. One hour after plating, the medium was changed, and Epo (100 ng/ml) or control buffer was added to the myocytes in a blinded fashion 30 min before apoptosis was triggered by staurosporine (0.1 μM; Sigma). After 16 h of incubation, cardiomyocytes were fixed and processed for *in situ* terminal deoxynucleotidyltransferase (TdT) detection of apoptosis (Roche Diagnostics). The number of TdT-labeled cells was determined by counting at least 500 myocytes in each culture dish and expressed as a percentage of the total number of cells. Omission of biotin-16-dUTP or TdT was used to generate negative controls.

Statistics. Unless otherwise indicated, all results are displayed as means $\pm$ SEM of replicates. One-way analysis of variance followed by Dunnett's test or the nonparametric Kruskal–Wallace analysis were used for statistical evaluation as appropriate.

Results

Evidence for a Heteromeric Complex Consisting of EpoR and βcR. Working on the assumption that tissue protection is mediated via a typical glycosylated cytokine receptor, adult rat brain, kidney,

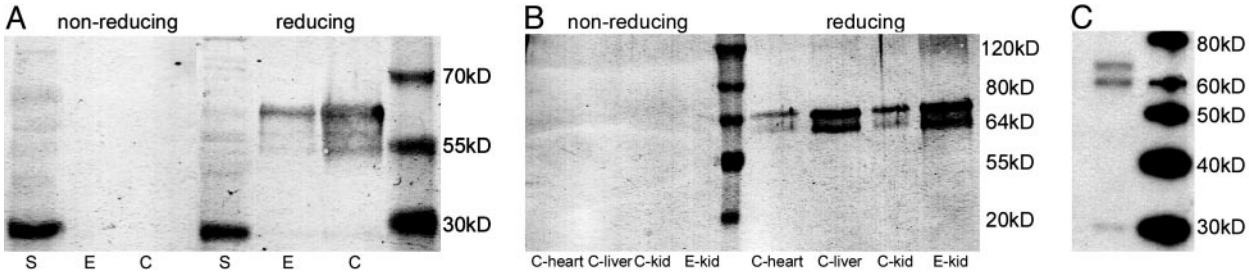

Fig. 1. Despite having no affinity for CEpo, EpoR is bound to a CEpo affinity column but within a complex. (*A*) Rat brain membrane proteins sequentially purified over a lentil lectin column and Epo or CEpo affinity columns were subjected to EpoR Western blotting in either a reduced or nonreduced state. Bands ($\approx$64 kDa) consistent with the EpoR were visualized only under reduced conditions. S, soluble EpoR-positive control (29 kDa); E, Epo affinity column; C, CEpo affinity column. (*B*) Membranes prepared from heart, kidney (kid), and liver show results similar to those for brain membranes but as a distinct doublet. (*C*) In contrast, cell membranes obtained from TF-1 cells and run under nonreducing conditions show the doublet EpoR.

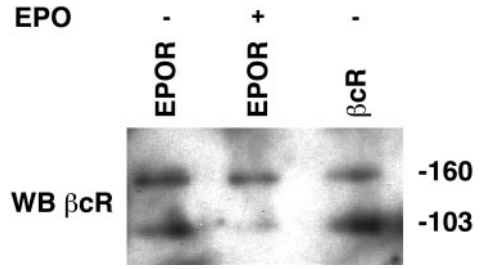

Fig. 2. EpoR and βcR are present as a complex in neuronal lysates. Immunoprecipitation of membranes prepared from P19 mouse neuroblastoma cells demonstrate that either anti-EpoR or anti-βcR pulls down a protein consistent with βcR (≈130 kDa), as well as a smaller molecular species. Equivalent results were obtained in the presence or absence of Epo.

and liver membrane preparations were first glycoprotein-enriched by passage over a lentil lectin column and then exposed to either an Epo or CEpo affinity column. Immunoblotting of reduced or nonreduced eluted proteins from either column with an anti-EpoR antibody visualized a principal band of ≈64 kDa molecular mass, consistent with previous reports of EpoR (5), but only under reducing conditions (Fig. 1 *A* and *B*). In contrast, membranes obtained from TF-1 cells, which signal via the homodimeric $(EpoR)_2$, showed bands consistent with EpoR under both nonreducing (Fig. 1*C*) and reducing (data not shown) conditions. These data suggested that the tissue-protective receptor consisted of EpoR within a larger complex that displayed an affinity for the CEpo column.

EpoR and βcR have been previously described as a complex; by using immunoprecipitation, we determined this complex was present in neuron-like cells. Membranes were prepared from undifferentiated neuronal-like murine P19 cells with or without a brief Epo exposure and then immunoprecipitated by using a mixture of two antibodies against EpoR that were raised against different regions of the molecule. Western blotting of the precipitated proteins with anti-βcR displayed a dominant band at the appropriate molecular mass for the βcR protein (≈130 kDa), as well as another band at ≈100 kDa (Fig. 2). The presence of Epo during incubation and precipitation did not affect the results. Similar findings were obtained from membranes prepared from adult mouse heart (data not shown).

Because the tissue-protective receptor (with high affinity for

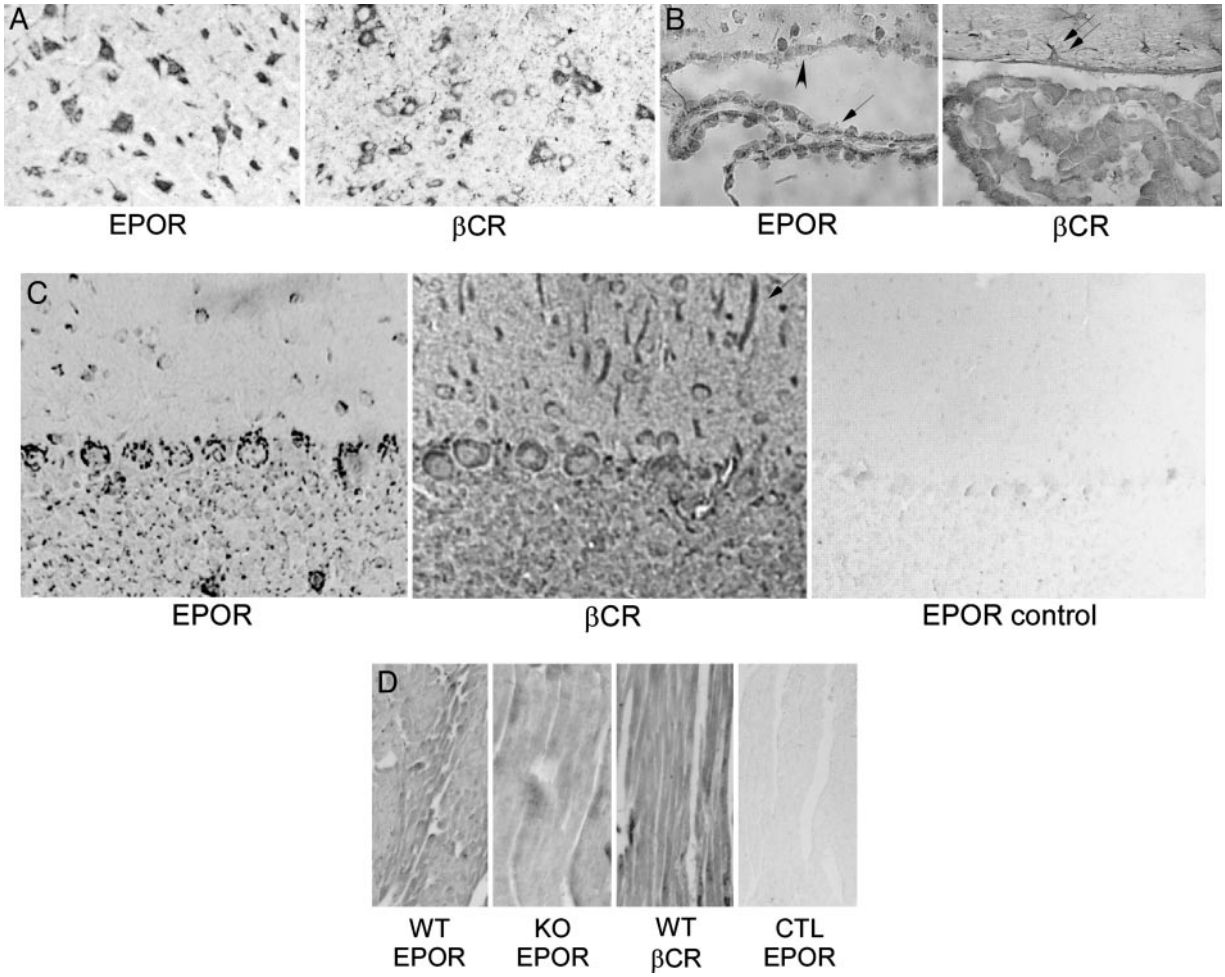

Fig. 3. Cells exhibiting tissue protection express both the EpoR and βcR subunit. (*A*) Spinal cord neurons within the central gray obtained from normal mice show prominent staining of the somata for both proteins. (*B*) Choroid plexus (arrow), as well as the ependymal cell layer (arrowhead), exhibit prominent staining for both EpoR and βcR. EpoR staining appears punctate. Radial glia (double arrow) are also doubly immunoreactive. (*C*) Purkinje cells of the cerebellum stain densely for EpoR in a punctate manner (*Left*), whereas anti-βcR stains somata, as well as the proximal dendrites (arrow), more diffusely. Note that neuronal somata in the molecular layer, as well as granule cells, are also immunopositive for both proteins. *Right* illustrates negative control (primary antibody omitted). (*D*) Sections of myocardium obtained from normal mice show prominent EpoR and βcR immunostaining. Sections obtained from the βcR knockout mouse exhibit prominent EpoR immunoreactivity. CTL EpoR is a negative control in which the primary antibody was omitted.

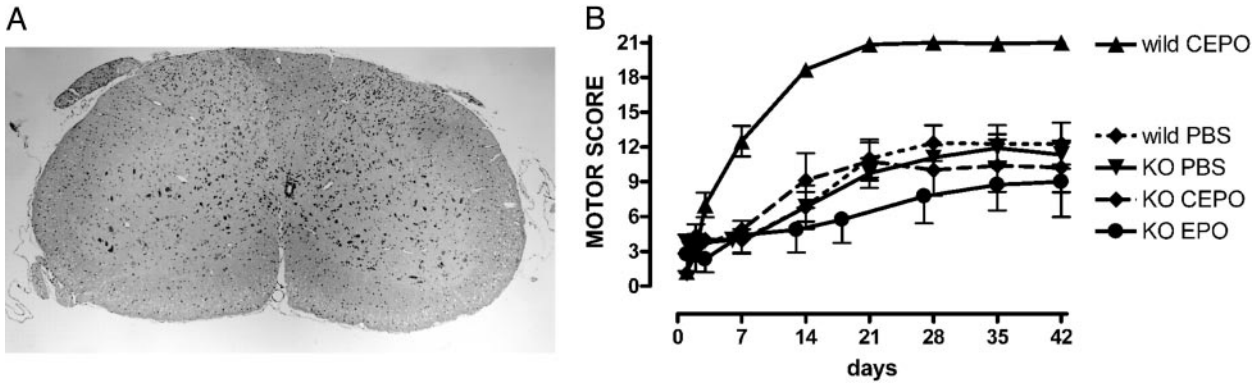

Fig. 4. CEpo restores motor function after spinal compression in wild-type mice but not in strain-matched βcR knockout mice. (*A*) Spinal cord morphology is normal in the βcR knockout mouse (hematoxylin/eosin-stained). (*B*) βcR knockout mice subjected to spinal cord compressive injury do not respond to either Epo or CEpo (10 μg/kg of body weight) given i.p. as a single dose immediately after injury.

CEpo) contained both EpoR and βcR, individual cells responsive to nonerythropoeitic tissue-protective cytokines should coexpress these proteins. In confirmation of this prediction, a heterogenous distribution of distinctive cell types expressing immunoreactivity for both βcR and EpoR was observed in normal rat tissues. For example, spinal cord central gray neurons (Fig. 3*A*) were immunopositive for both receptors. However, the subcellular localization of the proteins differed in large and small neurons within the brain and spinal cord: EpoR staining was localized predominantly to the neuronal somata in a punctuate cytoplasmic pattern, extending rarely into the proximal dendrites (e.g., the Purkinje cells within the cerebellum) (Fig. 3*C*). In contrast, although βcR immunoreactivity colocalized within the same neuronal type, it appeared extensively distributed throughout both the cell body and dendritic processes. In a noncomprehensive nervous system survey, prominent colocalization of βcR and EpoR also was observed in the choroid plexus, ependymal cells (Fig. 3*B*), and radial glia. In contrast, EpoR and βcR expression in other tissues appeared diffusely colocalized, e.g., in cardiac myocytes (Fig. 3*D*). In many regions examined, small capillaries were also positive for the two receptor proteins (data not shown). Two other antibodies against βcR and EpoR (obtained from R & D Systems) produced similar staining patterns (data not shown).

Studies in the βcR Knockout Mouse. If the βcR is a critical component of the tissue-protective receptor, the βcR knockout mouse should be unresponsive to tissue-protective cytokines. This mouse model is grossly normal phenotypically and is fertile. It is immunologically abnormal, especially within the eosinophil lineage, and ultimately develops a progressive pulmonary fibrosis with advancing age. The original descriptions of the young βcR knockout mouse reported no abnormalities of tissues and organs. We confirmed these observations by extensive microscopic examination of hematoxylin/eosin- and Nissl-stained sections that revealed no abnormalities of the βcR knockout brain, spinal cord (Fig. 4*A*), liver, kidney, or heart.

Spinal Cord Injury. Normal or βcR knockout male mice of 8–16 weeks of age received a moderate compressive lesion of the spinal cord, followed immediately by a single i.p. dose of Epo or CEpo (10 μg/kg of body weight) and were subsequently evaluated for motor function over 6 weeks. Mortality was similar between groups (≈10–20%). Wild-type mice responded to CEpo with a complete recovery within 4 weeks (Fig. 4). In contrast, βcR knockout animals exhibited no difference in motor function among the CEpo, Epo, or saline groups after 6 weeks. However, at earlier time points, βcR knockout animals receiving Epo exhibited a poorer motor recovery. Calculation of the area under the curve showed a significant difference between the Epo and PBS animals (133 ± 30 vs. 356 ± 36 motor-score days; $P < 0.05$). The simplified scoring of Tarlov (17) gave a similar outcome to the Basso scale (16). Bladder function was regained in parallel to the motor function (data not shown).

Primary Cardiomyocyte Survival. Cardiomyocytes prepared from the hearts of young wild-type or βcR knockout mice (8–12 weeks) were incubated in the presence of rhEpo (100 ng/ml) or control buffer and then exposed to staurosporine (0.1 μm). After a 16-h exposure, both the wild-type and βcR knockout cell cultures exhibited ≈70% apoptosis (Fig. 5) ($P < 0.001$). rhEpo added to the culture medium (100 ng/ml) improved survival, reducing apoptosis to ≈40% in the wild-type cells ($P < 0.001$). In contrast, βcR knockout cardiomyocytes did not respond to rhEpo.

Discussion

We have recently shown that the hematopoietic and tissue-protective activities of Epo are distinct and separate (6), implicating the existence of different receptors. Epo is a member of the cytokine superfamily type I that is characterized by pleotropic functionality. Receptors within this family often consist of heterogeneous combinations of proteins that can transduce different functions for the same ligand (e.g., gp130) (19). Despite our observations that CEpo does not bind to the EpoR dimer or monomer (6), there are several lines of evidence that firmly implicate the EpoR in tissue protection. First, under conditions of hypoxia or other metabolic stressors, brain cells greatly

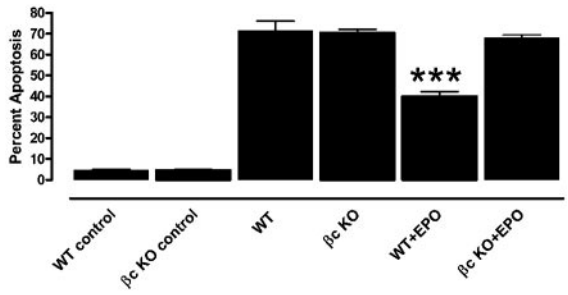

Fig. 5. rhEpo is tissue-protective of staurosporine-induced apoptosis for cardiomyocytes derived from wild-type cells but not identically prepared cells from βcR knockout mice. rhEpo was added (100 ng/ml) 30 min before the addition of staurosporine (0.1 μg/ml). Each condition corresponds to between four and eight replications. ***, $P < 0.001$ vs. staurosporine alone.

increase mRNA and immunoreactive EpoR (20, 21). Second, EpoR-neutralizing antibodies block neuroprotection (22). Third, EpoR knockout mice develop an abnormal brain and heart, characterized by massive cellular apoptosis. Fetal neurons can be cultured from the EpoR knockout embryos, and these display an increased sensitivity to stressors (e.g., ischemia) and do not respond to Epo (23).

As an initial approach to isolate the receptor with which CEpo interacts, we used a CEpo affinity column. Our preliminary work showed that an enrichment of glycosylated membrane proteins could be obtained if the membrane fraction was first passed over a lentil lectin column. Analysis of the proteins retained on the CEpo column showed that EpoR was indeed captured, despite exhibiting no affinity for CEpo. However, in this case, EpoR, unlike proteins prepared from TF-1 cells, was covalently bound to another protein (or proteins); it was only observable under reducing conditions (Fig. 1). The possibility that residual low levels of Epo contaminated the CEpo was eliminated by the demonstration that the CEpo used (tested up to 10 μg/ml) possessed no erythropoietic activity in the TF-1 and UT-7 bioassays.

Further, proteins from membranes prepared from the neural-like P19 cells, immunoprecipitated by using anti-EpoR antibodies, and immunoblotted by using anti-βcR, a receptor reported to be associated with EpoR (7), showed a band of a size consistent with βcR ($\approx$130 kDa) but, again, only under reducing conditions (Fig. 2). An additional molecular species that could be a variant or breakdown product of βcR also was observed. Immunocytochemistry further supported the hypothesis of the tissue-protective receptor consisting of EpoR and βcR proteins, because these proteins appear colocalized in those cells (e.g., neurons) for which CEpo is tissue-protective *in vitro* and *in vivo* (Fig. 3).

Taken together, the colocalization and binding data suggest that tissue protection signals through the interaction of Epo or CEpo with an EpoR–βcR heteromer. To test this hypothesis, we used a mouse model that lacked the βcR but was otherwise normal with respect to red cells and platelets, verifying a preserved hematopoietic action of Epo via the homodimer $(EpoR)_2$. As expected in the strain-matched, wild-type animals, tissue-protective cytokines were fully active in a spinal cord injury model, confirming that mice are effectively protected from damage, as previously shown for the rat (6). In contrast, both CEpo and Epo did not protect βcR knockout mice from compressional spinal cord injury (Fig. 4), although the histology of the uninjured spinal cord was normal. Immunohistochemical examination of the brain and spinal cord for EpoR confirmed expression levels comparable to those of wild-type tissues, making it unlikely that the loss in efficacy for Epo or CEpo was explained by reduced or absent expression of EpoR.

The significantly reduced area under the curve for Epo compared with CEpo in the spinal cord injury model in βcR knockout mice is interesting and could depend on unopposed actions of Epo through the classical $(EpoR)_2$. Particularly, platelet activation within the microvasculature (24) might delay or impair early recovery through development of microinfarctions. The βcR knockout model will be useful for distinguishing between the classical and tissue-protective effects of Epo.

βcR knockout mice have been shown to exhibit defects in eosinophil and macrophage populations, which could well affect the rate of recovery from spinal cord injury. To eliminate this possibility, as well as to examine the involvement of the βcR receptor in cytoprotective activities outside of the CNS, we used an *in vitro* model of primary cardiomyocytes obtained from the adult heart. In agreement with previous studies (25), Epo protects wild-type primary cardiomyocytes from staurosporine-mediated apoptosis (Fig. 5). In marked contrast, Epo had no cytoprotective effects on identically treated cardiomyocytes isolated from βcR knockout mice, despite the presence of abundant EpoR immunoreactive protein. In sum, these experiments are fully supportive of a role for βcR in the tissue-protective signaling of Epo and CEpo.

It is notable that the βcR knockout mice appeared histologically normal and did not appear to be more sensitive to injury (e.g., after exposure to staurosporine), as we would have predicted in the absence of a tissue-protective receptor. This lack of amplification of injury could depend on an additional IL-3-specific common β subunit highly homologous to βcR that is present only in the mouse. In the mouse, therefore, the βcR knockout will affect only GM-CSF and IL-5 signaling (26), not IL-3, which has itself been reported to possess tissue-protective properties. Notably, IL-3 and its receptors (both α and β) are locally made (27, 28) and act in a neurotrophic and neuroprotective manner (29, 30), including protection from spinal motor neuron transection (31). To answer this question, additional experiments using gene-silencing technology will need to be performed in another species, e.g., in the rat, for which abundant data relevant to tissue protection have been collected. If the IL-3-specific β subunit does confer protection, the role of tissue-protective cytokine receptors in the setting of injury may be even more dramatic than has been shown in these experiments.

Although the precise protein interactions of the EpoR and βcR have not been determined, they are likely homologous to the GM-CSF:βcR stoichiometry, because receptor assembly in this cytokine family occurs through highly conserved structural and chemical mechanisms (32). In this system, the ligand GM-CSF displays negligible affinity for the GM-CSF receptor or βcR alone (33), similar to what we have observed for CEpo. In the presence of the ligand, however, a high-affinity receptor complex consisting of a 1:1:2 ratio of GM-CSF:GM-CSF receptor:βcR is formed (33). Because at least four cytokines appear to use the βcR, multiple signaling possibilities exist in tissues expressing different α receptors and clearly require further study. For example, previous investigators have described a hierarchy in signaling, presumably based on differences in affinity of βcR to the different α receptor subunits (33).

Finally, although our experimental results identify βcR and EpoR as components of the receptor mediating tissue protection, further study will be required to understand many critical aspects of this association, e.g., whether other proteins also are members of the complex, the precise stoichiometry of the receptor subunits, and the signaling pathways. With this knowledge, rational development of tissue-protective cytokines can be initiated.

We thank Ulf Andersson Ørom for technical assistance, Dr. Michael Yamin for critically reading the manuscript, and Drs. L. Robb, C. G. Begley, J. A. Whitsett, and W. Hull for providing the βcR knockout mice. This work was supported in part by the Kenneth S. Warren Institute and by Grants RBAU01AR5J and Fondo Integrativo Speciale per la Ricerca-Neurobiotecnologie from the Ministero della Istruzione, Università e Ricerca, Rome (to P.G.).

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