Expression of the erythropoietin receptor in human heart

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Additional figures and an appendix are available online.

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Erythropoietin (EPO), the kidney hormone regulating erythrocyte production, activates the erythropoietin receptor (EPOR), resulting in antiapoptosis. To investigate the clinical significance of EPOR expressed in neuronal cells of the brain, EPO was administered to patients within 8 hours of the onset of stroke symptoms, which ultimately resulted in the reduction of cerebral infarct size and improvement of functional neurologic performance.¹

Rodents treated with EPO in an animal model of myocardial ischemia and infarction recently demonstrated superior myocardial function.^{2,3} The expression of the EPOR was shown for a variety of rodent and rabbit primary and permanent cardiomyocyte cell lines. But, as noted by several investigators,^{2,3} proof of the presence of the EPOR in the adult human heart is missing.

To overcome this deficit, we investigated adult human ventricular and atrial tissue for the expression of the EPOR.

Methods

The study was approved by the institutional review board, and written informed consent was obtained from all participants. In patients with severe aortic valvular stenosis (valve area < 0.7 cm²), ventricular tissue was obtained from the muscular septum obstructing the left ventricular outflow tract (Morrow procedure) and snap-frozen for RNA and protein analysis or formalin-fixed for immunohistologic analysis (n = 4 per group). Right atrial tissue from the site of the venous cannulation was processed accordingly. Samples were analyzed with reverse transcriptase-polymerase chain reaction, Western blot, and standard or double immunohistochemistry as described in the online supplement.

Results and Discussion

In this report we provide evidence of the expression of the EPOR in adult human ventricular and atrial tissue. Western blots (Figure 1) and reverse transcriptase-polymerase chain reaction (Figure E1) from human ventricular and atrial tissue homogenates indicate the presence of the EPO receptor in the human heart.

Next we elucidated which cells of the human heart express the EPOR. By using standard (anti-EPOR antibody) and double immunohistochemistry (cardiomyocyte-specific α -actinin antibody in combination with anti-EPOR antibody), we showed that ventricular myocytes (Figures 2 and E2, A-C) are markedly positive for EPOR. These results not only confirm the findings from animal experiments^{2,3} but also expand them to the adult human situation. A novel finding of this investigation was the presence of EPOR in human atrial myocytes (Figure E2, D, E), which had not been described to date. In addition, we confirmed that endothelial cells in the adult human heart are positive for EPOR (Figures 2 and E2, D). In contrast with a recent study on primary rabbit cardiac fibroblasts,⁴ EPOR expression in connective tissue of the adult human ventricle (Figure E2, C) or atria (Figure E2, D, E) was not observed. This discrepancy might reflect the differences between cell culture and in vivo conditions or species-specific differences. The observed subcellular distribution of the EPOR signal suggests that the EPOR is localized to the cell membrane (Figure E2, B, insert 1) and cytoplasm (Figure E2, B, insert 2). Although

Figure 1. Proof of EPOR expression in human heart. Western blot probed with antihuman EPOR antiserum (sc-695) gave a clear band at the expected molecular size. Lysate from the K-562 cell line served as positive control. *EPOR*, Erythropoietin receptor; α -*tubul*, α -tubulin.

the methods used do not provide final topologic proof of the cytoplasmic localization of the EPOR, it was recently reported that the EPOR indeed is degraded by internalization and subsequent targeting for lysosomal disposal. Recently, several investigations using animal experiments of myocardial ischemia or infarction reported significant improvements of myocardial function on treatment with EPO. Because a proof of concept study in humans has yet to be performed,³ our findings of the EPOR expression in the human heart are particularly significant. In this context of EPO-induced cytoprotection, it is remarkable that Leist and coworkers⁵ were able to dissect the hematopoietic activity from the cytoprotective activity. Thus, it might be possible to exploit the cytoprotective effect of EPO without the potentially dangerous side effect of polycythemia.

Our findings provide primary data evidence of the presence and localization of the EPOR in the adult human heart. They encourage investigation of the potential of EPO administration for myocardial protection in humans.

Figure 2. Identification of the EPOR positive cells in the human ventricular myocardium by immunohistochemistry. Ventricular myocytes (arrows) and endothelial cells (arrowheads) were positive (red-brown color) for EPOR. Chromogen AEC (Dakocytomation, Carpinteria, Calif), scale bar 10 μ m.

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Appendix E1

Methods

RNA extraction and semiquantitative reverse transcriptase-polymerase chain reaction. Reverse transcription of RNA and amplification of cDNA were performed in a thermal cycler (Whatman Biometra, Florham Park, NJ). For cDNA synthesis, reverse transcription was performed with oligo-dT priming and Omniscript (Qiagen, Hilden, Germany) according to the manufacturer's protocol. Polymerase chain reaction was performed in a thermal cycler (MWG-Biotech, Ebersberg, Germany) under the following conditions: 10 mmol/L Tris-HCl (pH 9.0), 50 mmol/L KCl, 1.5 mmol/L MgCl₂, 200 μ mol/L of each dNTP, 0.2 μ mol/L upstream and downstream primers, 2% reverse transcriptase mix, and 0.1 U/ μ L SuperTaq (Ambion Inc, Austin, Tex). Amplification conditions were 1 minute at 94°C, 1 minute at 56°C (erythropoietin receptor [EPOR]) at 64°C (β -actin), 2 minutes at 72°C for 30 cycles, and a final extension step of 10 minutes at 72°C. Primer pairs were as follows:

5'-EPOR 5'-GGCAGTGTGGACATAGTGGC-3'

3'-EPOR 5'-AGCAGGATGGATTGGGCAGA-3'

5'- β -actin 5'-TGACGGGGTCACCCACACTGTGCCCATCTA-3'

3'- β -actin 5'-CTAGAAGCATTTCGCGGTGGACGATGGA GGG-3'

Western blot. Tissue was homogenized in lysis-buffer (60 mmol/L Tris-HCl; pH 6.8, 10% glycerol, 1% Triton X-100, Protease Inhibitor Cocktail [Calbiochem, Darmstadt, Germany]), and protein extracts were separated on a 10% sodium dodecyl sulfate–polyacrylamide gel and transferred to nitrocellulose membranes (Hybond, Amersham Biosciences, Buckinghamshire, UK). Protein transfer was controlled by Ponceau S staining according to the manufacturer's protocol (Sigma-Aldrich, St Louis, Mo). Immunodetection was performed with rabbit polyclonal anti-EPOR antibody C-20 (1:100, sc695 Santa Cruz Biotechnology, Santa Cruz, Calif). A lysate of K-562 cell (sc2203, Santa Cruz Biotechnology) served as positive control, and the specificity of the antiserum was confirmed by co-incubation with blocking peptide (sc-695p, Santa Cruz Biotechnology, data not shown). Anti- α -tubulin (1:1000, Santa Cruz Biotechnology) and anti- β -actin antibody (A5691, Sigma-Aldrich) were used as loading controls. Incubation with primary antibody was followed by incubation with a 1:3000 dilution of goat anti-rabbit or goat anti-mouse horseradish peroxidase-labeled antibody (BioRad, Hercules, Calif) and visualization with enhanced chemiluminescence (Amersham Biosciences).

Histology. Ventricular and atrial-auricular tissue were immediately fixed in 4% buffered formalin (pH 7.4), processed rou-

tinely, and embedded in paraffin using an automatic tissue processor (Leica TP 1020, Leica, Heidelberg, Germany). Embedded tissues were serially sectioned on a Micron microtome (HM 440E, Micron, Neuss, Germany). The sections were examined with a Zeiss photomicroscope (Carl Zeiss Surgical, Inc, Thornwood, NY).

Immunohistochemistry. Immunohistochemistry was performed on 2- μ m-thin sections. Epitopes were retrieved by heat-induced antigen retrieval. Endogenous peroxidase activity was quenched with 3% hydrogen peroxide. Sections were blocked (CSA-Kit K1500, DakoCytomation, Carpinteria, Calif) and incubated with anti-EPOR antiserum (sc695, recognizes C-terminus, Santa Cruz Biotechnology, or 07-311, recognizes N-terminus, Upstate, Lake Placid, NY) or cardiac muscle-specific anti- α -actinin (A7811, Sigma, St Louis, Mo) for 15 minutes at room temperature. Coincubation of antiserum and blocking peptide (1:1, sc695:sc695P, Santa Cruz Biotechnology) and isotype-specific immunoglobulin-G antibody, respectively, served as negative controls (results not shown). The primary antibody was linked to an amplification system (CSA or Envision, DakoCytomation). Chromogen development with AEC, FastRed, and DAB (DakoCytomation), respectively, was followed by counterstaining with Mayer's hemalaun (Merck, Darmstadt, Germany).

Figure E1. EPOR mRNA expression in human heart. Agarose gel of reverse transcriptase-polymerase chain reaction showing the presence of EPOR mRNA in ventricular and atrial tissue. *Atri*, Atrium; *EPO*, erythropoietin.

Figure E2. Identification of the EPOR positive cells in the human heart by (double) immunohistochemistry. **A, B, C,** Ventricular myocytes (arrows in **A** and **B**) and endothelial cells (arrowheads in **A**) were positive for the EPOR. Double staining (**C**) for ventricular myocyte-specific sarcomeric α -actinin (dark brown) and EPOR (red) clearly showed that the EPOR was present in myocytes, but not in fibrocytes. The EPOR was found clustered to the sarcolemma (insert 1 in **B**) and more evenly distributed in myocytes (insert 2 in **B**). **D, E,** Atrial myocytes (arrows) and endothelial cells (arrowheads, insert 4 in **D**) expressed the EPOR. **F,** Negative control. The anti-EPOR antiserum recognized the C-terminal portion (sc-695) in (**A**) and (**D**), and the N-terminal amino acids 1-15 (07-311) in (**B**, **C**, and **E**). *n*, Nucleus; *fibro*, fibrocytes. Scale bar 10 μ m.