Functional and immunochemical characterisation of different antibodies against the erythropoietin receptor

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Abstract

Since it was discovered that the hematopoietic hormone erythropoietin (EPO) exerts neuroprotective effects in the CNS, many studies on the EPO receptor (EPOR) function and localisation in the CNS have been performed. For this purpose, commercially available anti-EPOR antibodies have often been applied. As the literature data on these antibodies show inconsistencies, we here systematically compared six frequently used, commercially available EPOR antibodies for different applications. Five of the antibodies appeared to specifically recognize recombinant rat and human EPOR in HEK293 cells by Western blotting, but the same antibodies yielded different and inconsistent results when human UT-7 cells or rat brain tissue were applied. Immunocytochemical staining of EPOR-transfected HEK cells only produced consistent results with three of the six antibodies. All antibodies stained neurons in rat brain sections, but with large differences in the staining pattern and only the C-20 EPOR antibody was found to label astrocytes. Since EPOR antibodies have been applied in several studies as EPOR antagonists, we further tested the antibodies for their capacity to functionally block the EPO–EPOR interaction in a cellular signalling system with STAT-5 phosphorylation as readout. Here, only the MAB307 antibody showed a partial effect at concentrations of 5–50 µg/ml.

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1. Introduction

The EPOR is known to mediate hematopoietic effects of EPO in the bone marrow, however, EPOR expression has also been reported to occur in various parts of the brain, where it is believed to mediate beneficial neurological effects (Brines and Cerami, 2005; Richmond et al., 2005). Indeed, numerous studies have shown an important neuroprotective and neurotrophic role of EPO in the brain during CNS development and pathogenesis (Brines and Cerami, 2005). The neuroprotective role of the EPOR has, however, been questioned by the finding that certain derivatives of EPO display neuroprotective properties similar to EPO, although these derivatives do not activate the classical EPOR (Leist et al., 2004; Savino et al., 2006). In light of this, determination of the expression pattern of the EPOR in the brain before and after brain injury may provide valuable information

on the role of this receptor in neuroprotection *in vivo*. Indeed, a series of studies investigating localisation and modulation of EPOR expression in the brain have been published, and it has been reported that the expression of EPOR in the brain of both rodents, sheep and humans is increased after ischemic insults (Bernaudin et al., 1999; Castillo-Melendez et al., 2005; Sairanen et al., 2006; Siren et al., 2001; Sun et al., 2004; Sola et al., 2005; Wen et al., 2004). These studies often rely on the use of commercially available anti-EPOR antibodies to provide information by immunohistochemistry and immunoblotting. However, we have in our studies become aware that immunohistochemical mapping of EPOR localisation produce diverging results when investigated with different anti-EPO antibodies. We thus sought to investigate the specificity and staining profiles of different anti-EPOR antibodies by using Western blotting and immunocytochemistry on overexpressed and endogenous EPOR, as well as immunohistochemistry on rat brain sections. The EPOR is a type 1 cytokine receptor with a single membrane-spanning domain (D'Andrea et al., 1990). In this study we have compared antibodies that are directed against either the intracellular C-terminal or the extracellular N-terminal part of the receptor. Western blots

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have been carried out on membrane preparations from cells or tissue in order to limit detection to the mature membrane-bound form of the EPOR and avoid detection of the smaller soluble EPOR (Kuramochi et al., 1990).

In addition to immunostaining and immunoblotting, anti-EPOR antibodies have been applied in several studies as apparent functional EPOR (Akimoto et al., 2000; Campana et al., 1998; Keswani et al., 2004; Lee et al., 2004; Liu et al., 2006; Martinez-Estrada et al., 2003; Park et al., 2006; Ruscher et al., 2002; Signore et al., 2006; Stuckmann et al., 2003). Antagonistic properties of these antibodies have, however, not been validated, hence we examined here the blocking effects of these antibodies on EPOR signalling.

2. Materials and methods

2.1. Antibodies

Six different antibodies directed against the EPOR were used for immunoblotting, immunohistochemistry, immunocytochemistry and assays for EPOR blockade. The antibodies used are listed in Table 1. Anti-EPO antibody (MAB287) used in UT-7 signalling assays was from R&D Systems (Minneapolis, USA).

2.2. Generation of EPOR overexpressing cells

The human *Epor* gene was amplified from human brain RNA using standard RT-PCR procedures and cloned into pCI-neo using EcoRI and XbaI. The 5' end of the coding sequence of the rat *Epor* (nucleotides 1–840) was amplified from total RNA purified from PC12 cells by RT-PCR and inserted into the IMAGE clone AI711156 containing the remaining coding sequence using EcoRI and the internal BglII site. The entire coding sequence was amplified from this construct using standard PCR and was inserted into pCI-neo using EcoRI and XbaI. DNA was transiently transfected into HEK293 cells using lipofectamine 2000 (Invitrogen, CA, USA) according to manufacturer's instructions.

2.3. Immunoblotting against EPOR

Western blot analysis was performed on membranes prepared from UT-7 cells, HEK293 cells transfected with the rat or human *Epor* construct, and from adult Sprague–Dawley rat brain. Cells or tissue were homogenized in 50 mM Tris, 1 mM EGTA, 5 mM MgCl₂, pH 7.4 supplemented with a protease inhibitor cocktail for mammalian cell and tissue extracts (Sigma–Aldrich, St. Louis, USA). Cell membranes were isolated as described elsewhere (Helboe et al., 1997). For whole cell lysates, the lysis buffer used was: 1% Triton X-100, 50 mM Tris, 150 mM NaCl, 2 mM EGTA supplemented with protease inhibitor cocktail (complete from Roche). Five µg (HEK293 cells), 10 µg (UT-7 cell membranes), 20 µg (UT-7 whole cell lysates) or 25 µg (rat brain) of total protein was loaded per well and separated by SDS-PAGE on a Bis–Tris gel (NuPAGE, Invitrogen). Immunoblotting was carried out using a panel of anti-EPOR antibodies (see Table 1), horseradish peroxidase-conjugated secondary antibodies (anti-rabbit, Sigma–Aldrich; anti-mouse, anti-sheep and anti-goat: DAKO, Glostrup, Denmark) and the enhanced chemiluminescence (ECL-Plus, Amersham, Little Chalfont, UK). Rainbow molecular weight markers (Amersham) were included on the blot.

2.4. Immunocytochemistry

Cells were grown in plastic chamber slides and fixed with 4% phosphate buffered paraformaldehyde for 10 min. Cells were then washed intensively in PBS. Antibodies were used at optimal dilution in PBS/1% BSA/0.1% Tween-20 and incubated for 1 h at room temperature (see Table 1 for dilutions). Alternatively, unfixed cells were incubated with primary antibodies in the absence of Tween-20 to evaluate cell surface expression of EPOR. After washing, cells were incubated for 1 h at room temperature with Alexa Fluor 488-conjugated secondary antibody (1:250; Molecular Probes, Eugene, OR).

Table 1
Anti-EPOR antibodies

Antibody	Source	Dilution WB	Dilution ICC	Dilution IHC	Epitope	Epitope localisation
M-20, #sc-697 (rabbit, polyclonal)	Santa Cruz	1:400	1:500	1:4000 + TSA	Carboxy terminus peptide of murine EpoR	Intracellular
C-20, #sc-695 (rabbit, polyclonal)	Santa Cruz	1:400	1:500	1:2000	Carboxy terminus peptide of human EpoR	Intracellular
H-194, #sc-5624 (rabbit, polyclonal)	Santa Cruz	1:200	1:500	1:1000	Amino terminus peptide (aa 21–214) of human EpoR	Extracellular
E3455-22 (sheep, polyclonal)	Nordic Biosite AB	1:500	1:500	1:500	Sequence from the extracellular domain of EPOR	Extracellular
AF-322-PB (goat, polyclonal)	R&D Systems	1:500	1:500	1:1000 + TSA	Total soluble recombinant human EPOR	Extracellular
MAB307 (mouse, monoclonal)	R&D Systems	1:200, 1:500	1:100	1:1000	Extracellular domain of human EpoR	Extracellular

WB: Western blotting, IHC: immunohistochemistry, ICC: immunocytochemistry, TSA: tyramide signal amplification. Santa Cruz Biotechnology, Santa Cruz, CA; Nordic Biosite AB, Täby, Sweden; R&D Systems, Minneapolis, MN.

2.5. Immunohistochemistry

Animal experiments were conducted in accordance with the Guide for the Care and Use of Laboratory animals, as promulgated by the National Institute of Health, and Danish laws. Adult male Sprague–Dawley rats were anaesthetised with Avertin and perfused via the ascending aorta with PBS/heparine followed by 4% phosphate buffered paraformaldehyde. The brains were embedded in paraffin and cut into 4 μ m sections. Sections were deparaffinised and boiled in 10 mM citrate buffer pH 6 in a microwave oven. Prior to immunostaining, endogenous peroxidase was inactivated with 1.5% H_2O_2 and sections were preincubated in 5% normal swine serum. The sections were incubated overnight at 4 °C with each of the 6 different anti-EPOR antibodies (see Table 1 for details and dilutions) followed by biotinylated secondary anti-rabbit (1:500; DAKO), anti-mouse (1:500; DAKO), or anti-sheep/goat (1:200, Amersham) antibodies at room temperature for 1 h and streptavidin–biotin-complex (Vector, Burlingame, CA, USA) for 1 h. Finally, immunostaining was visualised with 0.05% diaminobenzidine and 0.01% H_2O_2 in PBS. For each antibody the immunostaining was optimised by testing a range of dilutions and also including further amplification by applying biotinylated tyramide (Perkin-Elmer, Boston, MA). Data are shown for the lowest concentration of antibody yielding a positive staining. In parallel, immunohistochemistry was performed on 40 μ m free-floating cryo-sections and was shown to result in a staining pattern comparable to that obtained in paraffin sections.

2.6. UT-7 cells

For study of EPOR signalling we used the human hematopoietic cell line UT-7 (Komatsu et al., 1991). Cells were grown in RPMI 1640 with 10% fetal calf serum, 5 ng/ml recombinant human EPO (Dragon Pharmaceuticals, Vancouver, Canada), 100 U/ml penicillin and 100 μ g/ml streptomycin. Before experiments, cells were washed and starved overnight in starvation medium (RPMI 1640, 1% fetal calf serum, 100 U/ml penicillin and 100 μ g/ml streptomycin). For stimulation of UT-7 cells, recombinant human EPO was used in all experiments.

2.7. STAT5 phosphorylation assay

For induction of STAT5 phosphorylation, the UT-7 cells were stimulated for 15 min with 30 pM EPO. Anti-EPOR antibodies were preincubated with the cells for 30 min prior to stimulation with EPO and the anti-EPO antibody was preincubated with EPO for 30 min before addition to the cells. The maximal applied concentration of each antibody is equivalent to a 1:10 dilution of the stock solution. After stimulation, cells were spun down and lysed on ice for 30 min in lysis buffer (1% Nonidet P-40, 20 mM Tris–HCl pH 8, 137 mM NaCl, 10% glycerol with the following inhibitors: 1 mM Na_3VO_4 , 4 mM iodoacetamide, 10 mM NaF, 1 mM AEBSF and protease inhibitor mix Complete from Roche). Lysates were spun down at $15,000 \times g$ for 10 min, and supernatants were used for Western blotting. Twenty micrograms of protein was loaded per

well on 4–12% gradient Bis–Tris NuPAGE gels for SDS-PAGE (Invitrogen). For immunoblotting, anti-phospho-STAT5 antibody from Cell Signaling (MA, USA, #9351, dilution 1:1000) and anti- β -actin antibody from Sigma (A5441, dilution 1:5000) were used together with horseradish peroxidase-conjugated secondary antibodies (DAKO) and the enhanced chemiluminescence (ECL-Plus, Amersham). SeeBlue prestained standards (Invitrogen) were included on the blots. Antibodies used for this assay were submitted to buffer-change using size exclusion G25 spun column chromatography as described elsewhere (Buus et al., 1995) in order to eliminate sodium azide and other potentially toxic substances present in the original buffers.

3. Results

3.1. Recognition of over-expressed human or rat EPOR by Western blotting

We first tested the ability of the antibodies to recognise the EPOR from over-expressing HEK293 cells in a Western blotting assay. Cells were transfected with either human or rat EPOR, in order to test for possible species-specific recognition of the EPOR. The antibodies M-20, C-20, H-194, E3455-22 and AF-322 all recognised a dominant protein band with a molecular weight between 50 and 75 kDa in cells expressing either the rat or the human EPOR (Fig. 1A and B). The MAB307 antibody did not label bands in either preparation, demonstrating that this antibody is not suitable for Western blotting (Fig. 1A and B). None of the antibodies produced bands in untransfected cells. Taken together, these data suggest that the antibodies M-20, C-20, H-194, E3455-22 and AF-322 recognize both rat and human recombinant EPOR in Western blots.

3.2. Recognition of endogenously expressed human EPOR by Western blotting

For testing the ability of the antibodies to detect endogenously expressed EPOR, we used membrane and whole cell preparations from the human hematopoietic cell line UT-7. When subjecting membranes or whole cell lysates from UT-7 cells to immunoblotting, a variety of proteins of different sizes were detected with each antibody despite optimised washing and blocking conditions (Fig. 1C and D). Several of the antibodies recognised various bands between 50 and 75 kDa, and the identity of the EPOR band could therefore only be determined by including a HEK-EPOR control on the blot. M-20 and C-20 both recognised bands at the size of the EPOR, however they also identified additional bands between 50 and 75 kDa, which did not correspond to the size of the EPOR. H-194 and the E3455-22 antibody produced several different bands, including some between 50 and 75 kDa, but none seemed to correspond to the size of the EPOR. Only very weak bands appeared in this area with the AF-322 and MAB307 antibodies, but with elongated exposure time, AF-322 seemed to recognise a band of the right size (Fig. 1C). The lack of consistency between the labellings in UT-7 cells indicates that even though antibodies show a clear and specific response in overexpressing cells, they should be used

Fig. 1. Recognition of the EPOR by anti-EPOR antibodies in Western blotting. Preparations of membrane proteins were separated by Western blotting and probed against different anti-EPOR antibodies. Membranes were prepared from HEK293 cells over-expressing either the human (A) or the rat (B) EPOR or from rat brain homogenate (B). U: untransfected HEK293 cells; T: EPOR-transfected HEK293 cells; B: rat brain homogenate. Western blots of whole cell lysates (C) or membrane preparations (D) from UT-7 cells known to express the EPOR endogenously. The outer right lane in the upper blot (C) shows lysate from HEK-293 overexpressing the human EPOR (blotted with M-20). Arrowhead marks the size of the EPOR in HEK293 cells. MW: molecular weight marker.

cautiously when aimed to detect endogenously expressed EPOR in cells and tissues. We suggest an EPOR control (i.e. HEK cells overexpressing the EPOR) is always included on blots.

3.3. Recognition of EPOR in rat brain homogenate by Western blotting

Since the EPOR has been reported to be expressed in rat brain, we used this tissue to test whether the antibodies detected specific bands of endogenously expressed brain EPOR. In samples of membranes prepared from total rat brain homogenate, the M-20 antibody detected a single protein of higher molecular weight than in HEK293 cells over-expressing the rat EPOR (Fig. 1B). This is most likely not the EPOR, but we cannot exclude the possibility that differences in molecular weight could be explained by tissue-specific glycosylation or processing of the EPOR. The C-20 and E3455-22 antibodies recognised two major bands, migrating above and below that of rat EPOR-transfected cells. However, none of these bands corresponded in size with each other or to the one detected by M-20. As in UT-7 cells, it is therefore not possible by Western blotting to identify one clearly defined band representing the EPOR. One potential explanation for the situation could be that the EPOR is expressed at very low levels in the normal brain. It was not possible to distinguish clearly labelled protein bands in rat brain preparations reacted with MAB307, AF-322 or H-194 (Fig. 1B).

3.4. Immunocytochemistry on HEK293 cells over-expressing EPOR

We further tested the antibodies for immunocytochemical staining of HEK293 cells transfected with either human or rat EPOR. The antibodies C-20, M-20 and MAB307 stained both rat and human EPOR-HEK293 permeabilised cells (Fig. 2).

Furthermore, MAB307 antibody stained cell surface EPOR as assessed in unfixed and non-permeabilised cells, confirming that the MAB307 clone recognises an extracellular epitope of the EPOR. C-20 and M-20 recognise intracellular epitopes and as expected failed to stain EPOR in unfixed and non-permeabilised cells (Fig. 2). H-194, E3455-22 and AF-322 antibodies failed to yield consistent staining in EPOR-HEK293 cells both permeabilised and non-permeabilised (data not shown). MAB307, M-20 and C-20 seemed to be EPOR specific in this system, as no staining was observed in non-transfected cells or when the primary antibody was omitted (data not shown).

3.5. Immunohistochemical staining of rat brain

We next investigated EPOR staining pattern and cellular localisation in the adult rat brain by use of the different antibodies for immunohistochemistry. Staining of cryosections and paraffin sections resulted in similar staining patterns, and only immunostainings obtained in paraffin sections are shown in Fig. 3. In all cases, no staining was present when the primary antibody was omitted. Staining data obtained with different antibodies showed broad immunoreactivity in neurons of telencephalon, diencephalon, and mesencephalon in the rat brain, and positively stained neurons were found in all areas of the cerebral cortex with all anti-EPOR antibodies used (Fig. 3A). However, staining patterns obtained with the different antibodies differed. While the C-20, H-194, MAB307, AF-322, and E3455-22 antibodies stained most neurons throughout the cortex (Fig. 3A), and the hippocampus (Fig. 3B), staining with the M-20 antibody showed an intense immunoreactivity restricted to a subset of neurons in these areas (Fig. 3A+B). MAB307 broadly stained cortical (Fig. 3A, MAB307), and hippocampal neurons (Fig. 3B, MAB307), as well as cells resembling oligodendrocytes within the corpus callosum (Fig. 3C, MAB307). White matter cells

Fig. 2. Immunocytochemical staining of EPOR-transfected cells. HEK293 cells were transfected with either the human or the rat EPOR and then stained with primary anti-EPOR antibodies and secondary fluorescent antibodies. The antibodies were applied both to fixed, permeabilised cells (+perm.) and unfixed, non-permeabilised cells (–perm.) Stainings with the H-194, AF-322 and E3455-22 antibodies did not yield consistent staining of EPOR transfected cells in our experiments.

were also labelled by AF-322, whereas the C-20 antibody was found to stain astrocytic cell bodies (Fig. 3C).

At the cellular level, staining also differed between the different EPOR antibodies. Immunostaining with E3455-22, MAB307, C-20 and AF-322 antibodies resulted in a diffuse staining of cytoplasm as well as some nuclear staining (Fig. 3A). M-20 staining was more distinct in the cytoplasm with a granular pattern, whereas H-194 yielded a pronounced punctuate and threadlike cytoplasmic staining (Fig. 3A). For all antibodies, elements of plasma membrane staining may be present, however, it was not possible to clearly distinguish this from the cytoplasmic reactivity. The C-20 antibody occasionally stained single neurons strongly (Fig. 3A+B), but broad neuronal staining was also observed, e.g. in the thalamic nuclei (Fig. 3C). In summary, we observed different patterns of tissue distribution and cellular localisation with the different antibodies, however, we found immunoreactivity to be predominantly in neurons.

3.6. Inhibition of EPOR signalling

Since anti-EPOR antibodies have been used as EPOR antagonists in mechanistic studies, we tested the ability of these six antibodies to block EPOR-mediated signalling in UT-7 cells. Signalling through the EPOR was detected as phosphorylation of the transcription factor STAT5, this being part of the EPOR signalling cascade activated by EPO (Wakao et al., 1995). Cells were preincubated with anti-EPOR antibodies and then stimulated with 30 pM EPO for 15 min at 37 °C before lysis and analysis by Western blot. It was confirmed that EPO clearly induced increased phosphorylation of STAT5 compared to the control. As a positive control for interruption of the EPO–EPOR interaction we used 10 µg/ml (67 nM) of an anti-EPO antibody, which caused a complete block of STAT5 phosphorylation (Fig. 4A). Among the anti-EPOR antibodies, only MAB307 which is directed against the extracellular domain of the EPOR showed some inhibition of EPO-induced STAT5 phosphorylation. Although this inhibitory effect was seen at 5 µg/ml (33 nM), even 50 µg/ml (330 nM) antibody did not completely abolish

STAT5 phosphorylation (Fig. 4B). We have observed that the MAB307 antibody does not block proliferation of UT-7 cells over a 48 h time-period at any concentration (data not shown), indicating that inhibition of the short-term EPOR-mediated signalling is not synonymous with a persistent blockade of the receptor. Furthermore, we have found in binding studies on UT-7 cells, that MAB307 does not block the binding of iodinated EPO to the EPOR (data not shown). Thus, MAB307 may trigger an allosteric conformational change of the EPOR resulting in partial inhibition of EPO-induced signal transduction. None of the remaining antibodies (H-194, M-20, C-20, E3455-22 and AF-322) blocked STAT5 phosphorylation (Fig. 4B–D) or EPO-mediated proliferation (data not shown). The M-20 and C-20 antibodies are directed against the C-terminal intracellular domain of the EPOR, and therefore are not expected to block EPOR activation. H-194, AF-322 and the E3455-22 antibody are directed against the extracellular domain of the receptor, but yet they failed to block EPO-mediated signalling in our assay. It has been shown that certain non-commercial EPOR antibodies could function as EPOR activators, inducing homodimerisation of the receptor (Schneider et al., 1997). However, the antibodies we have tested here were not found to activate signalling through the EPOR (Fig. 4).

4. Discussion

We have tested a panel of six different anti-EPOR antibodies for their ability to specifically detect human and rat EPOR by immunoblotting, immunocytochemistry and immunohistochemistry. In Western blotting, all antibodies except the MAB307 detected a single dominant band in HEK293 cells overexpressing the EPOR. Although the MAB307 did not detect EPOR in Western blots, this antibody was shown to block signalling through the EPOR and to stain non-permeabilised unfixed EPOR-transfected cells. This suggests that MAB307 recognises a conformational epitope, thereby making it unsuitable for Western blotting.

Fig. 3. Immunohistochemical staining of rat brain sections. Micrographs showing paraffin sections of rat brain immunohistochemically labelled with six different anti-EPOR antibodies. (A) Staining of sections from the neocortex. (B) Staining of sections from the hippocampal CA1 region. (C) Examples of variations in specific staining patterns with different antibodies. MAB307 intensely stained neurons in the CA3 region as well as oligodendrocyte-like cells in corpus callosum. C-20 showed intense immunostaining of dorsomedial thalamic nucleus as well as astrocytes in the optic chiasm. Scale bar: 25 μ m.

(Campana et al., 1998; Keswani et al., 2004; Lee et al., 2004; Liu et al., 2006; Martinez-Estrada et al., 2003; Park et al., 2006; Ruscher et al., 2002; Signore et al., 2006; Stuckmann et al., 2003). In four cases, unspliced Santa Cruz antibodies have been applied (Liu et al., 2006; Keswani et al., 2004; Ruscher et al., 2002; Stuckmann et al., 2003) and, surprisingly, in two cases the Santa Cruz M-20 antibody directed against the intracellular domain of the EPOR was applied as an EPOR antagonist (Martinez-Estrada et al., 2003; Signore et al., 2006). Based on the epitope specificities and our functional data from the STAT5 assay, it must be assumed that effects induced by application of these anti-EPOR antibodies are not necessarily due to blocking of the EPO–EPOR interaction. In this context, it should be kept in mind that antibodies are frequently stored in solutions containing up to 15 mM sodium azide. Azide may have substantial impact and toxic effects on cell cultures even at concentrations as low as 15 μ M (O’Riordan et al., 2000). Sodium azide can be avoided by applying a buffer change on the antibodies, as we have done here.

In summary, we encourage that these antibodies are used with caution, and that conclusions should not be drawn from the use of these antibodies alone. The production and thorough characterisation of new and more specific anti-EPOR antibodies is a major challenge for the future.

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