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Validation of Endothelin B Receptor Antibodies Reveals Two Distinct Receptor-related Bands on Western Blot

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Abstract

Antibodies are important tools for the study of protein expression, but are often used without full validation. In this study, we use Western blots to characterize antibodies targeted to the N- (NT) or C-termini (CT) and the second (IL2) or third intracellular (IL3) loops of the endothelin B receptor (ETB). The IL2-targeted antibody accurately detected endogenous ETB expression in rat brain and cultured rat astrocytes by labeling a 50kD band, the expected weight of full-length ETB. However, this antibody failed to detect transfected ETB in HEK293 cultures. In contrast, the NT-targeted antibody accurately detected endogenous ETB in rat astrocyte cultures and transfected ETB in HEK293 cultures by labeling a 37 kD band, but failed to detect endogenous ETB in rat brain. Bands detected by the CT-targeted or IL3-targeted antibodies were found to be unrelated to ETB. Our findings show that functional ETB receptors can be detected at 50 kD or 37 kD on Western blot, with drastic differences in antibody affinity for these bands. The 37 kD band likely reflects ETB receptor processing, which appears to be dependent on cell type and/or culture condition.

Keywords

Endothelin B receptor; antibody; Western; immunoblot

1. Introduction

Endothelin B receptors (ETB) are extensively studied due to wide spread expression and importance in a variety of physiological functions in cardiovascular, gastrointestinal, excretory and nervous systems [1; 15; 24]. Antibodies are an essential research tool for the localization and quantification of ETB expression through techniques such as immunohistochemistry and Western blot. These techniques rely on the affinity and

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specificity of the antibodies used; however, many studies employ antibodies that have not been fully validated. Identifying ETB expression using Western blot is further complicated by published reports that ETB can appear at either 50kD, the predicted molecular weight for the full length receptor, or at approximately 37kD, due to modifications such as N-terminal cleavage [8-11; 18]. There is also currently no knowledge regarding antibody cross-reactivity between these two different molecular weight forms.

In this study, we tested the ability of four commercially available antibodies targeted to different epitopes of the ETB receptor to detect endogenous and transfected ETB receptors using Western blot (Table 1). Primary rat astrocytes and rat brain homogenates were used as sources of endogenously expressed ETB, while native and ETB-transfected Human Embryonic Kidney cells (HEK293) were used as negative and positive controls, respectively [6; 17; 26].

2. Material and Methods

2.1 Cell culture

Primary rat astrocytes were purchased from Lonza (Bazel, Switzerland) and grown in astrocyte growth media according to manufacture's instructions. HEK293 cells, a generous gift from Dr. P. Hogan (Harvard Medical School, Boston, MA), were grown in DMEM (Invitrogen, Carlsbad, CA). Media for transfected HEK293 cells was supplemented with 500µg/mL geneticin (Invitrogen). All cells were cultured with 0.5% Penicillin/Streptomycin (Invitrogen) in a humidified incubator at 37°C and 5% CO₂. When down-regulation of ETB was required, astrocytes were treated with ET-1 (100 nM) and cycloheximide (20 µg/mL) by adding these reagents into their normal culture media. Samples were collected 24 hours after this treatment.

2.2 Western blot

Rat brain extract was purchased from Boston Bioproducts (Ashland, MA). Cultured cells were lysed for 10 min in ice-cold RIPA buffer containing 3% protease inhibitor (Sigma-Aldrich, St Louis, MO) and then centrifuged at 14,000 g for 20 min at 4°C. Supernatants were collected and stored at -80°C with protein concentrations determined using a BCA assay kit according to manufacturer's instructions (Pierce, Tewksbury, MA). Protein samples (15 µg/well in 25 % Laemmli buffer, Boston BioProducts) and kaleidoscope molecular weight ladders (Bio-Rad Laboratories, Hercules, CA) were separated by SDS-PAGE on Mini-PROTEAN TGX Precast Gels with a 4-15% gradient (Bio-Rad) for approximately 35 minutes at 150V in running buffer (25mM Tris base, 192mM glycine, 1% SDS, pH 8.3). Proteins were transferred to a PVDF membrane (Millipore, Billerica, MA) in transfer buffer (25mM Tris base, 192mM glycine, 20% methanol, pH 8.3) using a wet blotting method at 400 mA for 90 minutes. The membrane was blocked with 5 % Amersham ECL Blocking Agent (GE Healthcare, Buckinghamshire, United Kingdom) in tris-buffered saline and 0.05 % Tween-20 (TBST, Boston BioProducts) for one hour and then incubated with primary antibody (see Table 1) in TBST with 1 % blocking agent at 4°C overnight. This was followed by three 10-minute washes in TBST, a one-hour room temperature incubation with the appropriate horseradish peroxidase (HRP)-conjugated secondary antibody (Santa Cruz

goat anti-rabbit or donkey anti-goat, see Table 1) in 1 % blocking agent and then another three 10-minute wash in TBST. For chemiluminescence detection, membranes were exposed to SuperSignal West Dura Extended Duration Substrate (Biorad) and visualized using a Biorad ChemiDoc XRS chemiluminescence detector. After labeling with ETB-targeted antibodies, membranes were stripped for 60 minutes with Restore PLUS Western blot stripping buffer (Thermo Scientific) at room temperature, rinsed 3 times for 10 minutes/ rinse, re-blocked and incubated overnight at 4°C with a GAPDH-targeted antibody (Millipore, 1:20000). Membranes were processed for GAPDH as described above using goat anti-mouse secondary antibody (Santa Cruz, 1:20000).

Detection and quantification of Western band intensities was conducted using Quantity One software (Biorad). Bands were normalized to GAPDH by dividing the average intensity of the band by the average intensity of the GAPDH band from the same sample labeled on the same gel. These normalized intensity values were then calculated relative to control and statistically analyzed with Statistica software (Statsoft, Tulsa, OK) using ANOVA and Dunnett's post-hoc tests to determine which groups were significantly different from their respective controls.

2.3 qPCR

qPCR was conducted as described previously [19]. RNA was isolated from 3 separate passages of 80-100% confluent cultures using an RNeasy kit (Qiagen, Valencia, CA), after which 1µg of total RNA was synthesized to cDNA using an iScript kit (Biorad, Hercules, CA). cDNA was amplified using Evagreen™ PCR supermix on a miniopticon thermocycler (Biorad) using the following protocol: 94°C for 3 minutes, 45 cycles of 94°C for 30 seconds, 60°C for 30 seconds and 72°C for 1 minute, and then a melting curve (65°C to 95°C in 0.5°C increments) to confirm primer specificity. Two separate qPCR experiments were each conducted in duplicate on three culture samples for a total of 4 replicates per data point. Data were analyzed using BioRad CFX manager with ETB expression normalized to the housekeeping gene Cyclosporin-A, adjusting for primer efficiencies calculated using REST 2009 software (Qiagen) from a serial dilution of each primer. Samples prepared substituting reverse transcriptase with RNAase-free water failed to amplify any detectable product. Primer sequences for ETB and CycloA have been previously described [19].

2.4 Calcium imaging

Calcium imaging experiments were conducted as previously described [19]. Intracellular calcium was determined by excitation microfluorometry using an inverted IX17 microscope (Olympus America Inc., PA) equipped with a Lambda DG-4+ digital camera (Hamamatsu Photonics, Japan). Coverslips were loaded with fura-2AM (4µM, Invitrogen) in Ringer's solution (NaCl 155mM, KCl 4.5mM, CaCl₂ 2mM, MgCl₂ 1mM, D-glucose 10mM, Hepes 5mM, pH 7.4) for 30 minutes in the dark at room temperature. Intracellular calcium levels were determined by the ratio of fura-2 fluorescence using the following equation: $F/F_0 = (F - F_0)/F_0$, where F is the fluorescence light intensity at each time point and F₀ is the baseline fluorescence intensity averaged over 10s before the stimulus application. Responding cells were defined as those with >10% increase in F/F₀. ET-1 (30 nM) was added alone or in combination with BQ-123 (100 nM) or BQ-788 (100 nM) after respective

antagonist pre-treatment of 5 minutes. Data were analyzed by *t*-test to determine whether BQ-123 or BQ-788 significantly reduced ET-1 calcium responses.

2.5 [¹²⁵I]-ET-1 binding

Equilibrium binding studies were conducted on 90 % confluent cells in 12-well plates, 24 hours after seeding as previously described [19]. All binding studies were conducted in triplicate and performed at 4 °C to prevent receptor internalization. Cells were incubated for 3 hours in 1 mL of 0.1 % BSA in culture medium, with varying concentrations of [¹²⁵I]-ET-1 alone (saturation experiments) or in the presence of BQ-123 or BQ-788 (competition experiments). [¹²⁵I]-ET-1 in the supernatant and cell lysate were measured in Ecolite(+)TM Liquid Scintillation Cocktail (MP Biomedicals, Solon, OH) with a Beckman Model LS8500 (Beckman, Inc; Palo Alto, CA) scintillation counter for 5 minutes/sample. Specific binding was defined as the difference between total binding and nonspecific binding, the latter determined by co-incubation with 500 nM unlabelled ET-1. Protein concentration was measured using a BCA protein assay (Pierce Biotechnology, Rockford, IL). For the competition binding studies (Figure 1D), data were fit using a one-site non-linear regression analysis in Graphpad Prism (Graphpad Software Inc., La Jolla, CA).

2.6 Stable Transfection of HEK293 Cells

Expression constructs containing the entire ET_B coding sequence together with the neomycin resistance gene (generous gift from Dr. Oksche, Muldipharma Research, Germany) were sequenced to verify integrity of the coding sequence. Plasmid DNA was prepared for transfection using an endotoxin free purification kit (Qiagen). The day before the transfection, DNA vectors were enzymatically linearized and subsequently purified by ethanol precipitation. In addition, HEK293 parental cells were seeded onto 6-well plates to obtain 80% confluence the next day. Cells were then transfected using lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. About 16 hours post-transfection, the cells were transferred into a 10 cm dish and allowed to settle down overnight. The next day, the medium was exchanged for medium supplemented with 0.5 g/l geneticin (Invivogen). For the next 7-10 days the medium was replaced every day with fresh medium supplemented with geneticin until all the cells in the untransfected control had died. At this point clones were isolated by carefully dispensing trypsin over the isolated colonies and transferring each isolated clone into a single well of a 12-well plate containing medium supplemented with geneticin. Clones were isolated for each construct, amplified and stored in liquid nitrogen.

3. Results and Discussion

3.1 ETB expression in transfected HEK293

Native HEK293 were chosen as a negative control, as we have previously shown that these cells lack ETB expression [19]. Native HEK293 show almost no ETB mRNA and the low levels of [¹²⁵I]-ET-1 binding seen in these cells are concentration-dependently displaced by the ETA receptor-selective antagonist BQ-123 with an IC₅₀ consistent with ETA receptor binding. Native HEK293 also do not respond to ET-1 exposure with an increase in

intracellular calcium, likely due to minimal expression of ETA receptors that is below a threshold for triggering detectable release of intracellular calcium [19; 32]

Transfection of functional ETB in HEK293 cells was verified by qPCR, calcium imaging and radioligand binding (Figure 1). Transfection raised ETB mRNA expression by almost 800-fold (from 1 ± 0.29 to 797.76 ± 67.24). Calcium imaging demonstrated that transfected receptors couple to intracellular signaling pathways (Figure 1A and B). , and the robust increases in intracellular calcium were significantly reduced by pretreatment with the ETB receptor-selective antagonist BQ-788 (t-test, $p < 0.001$), but not the ETA receptor-selective antagonist BQ-123. HEK-ETB also showed a strong increase in [125 I]-ET-1 binding compared to native HEK293 (Figure 1C). [125 I]-ET-1 binding was dose-dependently displaced by increasing concentrations of the ETB receptor-selective antagonist BQ-788 with a calculated IC₅₀ of 165 ± 24 nM, (Figure 1D). Thus, HEK-ETB were successfully transfected with functional ETB receptors that were not present in native HEK293 cells.

3.2 Western blot for ETB

Protein samples from rat astrocytes, rat brain homogenate , native HEK293 and HEK-ETB were separated by SDS-PAGE electrophoresis, transferred to a PVDF membrane and labeled with primary antibodies targeted to different epitopes of the ETB (Table 1, Figure 2). Primary antibodies were used at 1:500, as prominent bands could be seen for all antibodies at this dilution (Figure 2). The IL2-targeted antibody labeled a band close to 50 kD, the expected molecular weight of full-length ETB protein. This band was present in brain, astrocyte and HEK-ETB, but not in the native HEK293 samples that lack ETB message or receptor expression (Figure 2A). Some much less intense bands could also be seen at molecular weights above 50kD in brain and astrocyte samples. The NT-targeted antibody intensely labeled a band at approximately 37 kD (Figure 2B). This band was present in astrocytes and HEK-ETB, but was absent from native HEK293 and brain.

The CT- and IL3-targeted antibodies both intensely immunolabeled bands at approximately 60 KD in brain, astrocyte and HEK-ETB samples, which were absent in native HEK-293 (Figure 2C and D). Bands near 50 kD could only be detected in some of the ETB-expressing samples and these bands were far less intense compared to the band seen near 60 kD. A band near 85 kD was also seen for both of these antibodies in native HEK-293. Numerous other immuno-reactive bands could be seen at molecular weights above 50 kD for both of these antibodies.

To determine whether bands identified by ETB-targeted antibodies represented some form of ETB receptors, samples were taken from astrocytes, known to express ETB, that were cultured with ET-1 (100 nM) and cycloheximide (20 μ g/mL) for 24 hours to down-regulate ETB receptors (Figure 3). Upon activation by ET-1, ETB receptors are internalized and targeted for degradation [3; 20]. Concurrent treatment with the protein synthesis inhibitor cycloheximide prevents synthesis of new ETB receptors to replace those that were degraded. This treatment has been shown previously to reduce ETB receptor expression, as analyzed by Western blot [20].

Intensity of the 50 kD band labeled by the IL2-targeted antibody was significantly reduced by treatment with ET-1/cycloheximide (Dunnett's test $p < 0.05$), as was the 37 kD band labeled by the NT-targeted antibody (Dunnett's test $p < 0.05$, Figure 3B). Importantly, both of these bands were significantly less intense for the ET-1 + cycloheximide co-treatment compared to cycloheximide alone (t -test, $p < 0.05$), indicating that these bands represented ETB receptors. Cycloheximide alone caused an insignificant reduction in the intensity of both these bands ($p = 0.63$ for 50 kD and 0.15 for 37 kD). ET-1 treatment alone also had no significant effect ($p = 0.15$ for 50 kD and 0.88 for 37 kD), likely due to replacement of internalized and degraded ETB with newly synthesized receptors. ETB receptors have a proposed role in ET-1 clearance, so a rapid replacement of these receptors may aid in this function [4].

The significant reduction in intensities of the 50 kD band detected by the IL2-targeted antibody and 37 kD band detected by the NT-targeted antibody following ET-1 + cycloheximide treatment, is strong evidence that these bands indeed represent ETB, although possibly different forms of the receptor. Interestingly, the tested antibodies had strikingly different affinities for these two forms of ETB. The IL2-targeted antibody strongly labeled the 50 kD form, but was unable to detect the 37 kD form. Conversely, the NT-targeted antibody strongly labeled the 37 kD form, but did not detect the 50 kD form. Thus, the ability of a primary antibody to detect ETB will be dependent on the form of ETB expressed by the sample and a combination of antibodies that recognize both forms may be required to accurately confirm or refute ETB expression. A similar problem may arise in immunocytochemical labelling of receptors, for which the combined results from several different antibodies may be necessary to validate the presence of ET receptors.

The 60 kD bands recognized by the 3rd IL- and CT-targeted antibodies, in contrast, do not appear to represent any form of ETB receptors. No difference in intensity was seen in response to ET-1/cycloheximide for the 60 kD band labeled by the 3rd IL-targeted antibody (ANOVA $p = 0.241$). Intensity of the 60 kD band labeled by the CT-targeted antibody was actually significantly increased with ET-1/cycloheximide treatment (Dunnett's test $p = 0.048$) compared to control and there was no significant difference between ET-1 treatment alone and ET-1/cycloheximide used in combination (t -test $p = 0.368$). The lack of a significant reduction in band intensity in response to ET-1/cycloheximide treatment indicates that these 60 kD bands are not specific to ETB.

Endothelin receptors are known to form homo- and heterodimers [7; 21-22; 30-31] and endothelin receptor dimerization has been shown to delay internalization, potentially limiting degradation by ET-1 + cycloheximide. However, it seems unlikely that this 60 kD band represents endothelin receptor homo- or heterodimers, since this molecular weight is less than the expected weights of dimers made from the 37 kD or 50 kD monomers.

The 37 kD band detected by the NT-targeted antibody in astrocytes and HEK-ETB is below the molecular weight predicted for the full-length ETB receptor. However, such a 37 kD band has been reported previously in HEK293 cells transfected with ETB receptors [9; 11; 20]. It is unclear what type of receptor processing resulted in the 37 kD band seen in astrocyte and HEK-ETB samples, but N-terminal cleavage has been shown to result in an

approximately 37 kD band on Western blot in ETB-transfected HEK293 cells, as well as vascular smooth muscle and Madin-Darby canine kidney cells [8-11]. The antibody that best labeled this band (NT) in our study was targeted to the N-terminus, but the proprietary nature of this antibody's exact epitope makes it uncertain whether this site would be removed by N-terminal cleavage or if, in contrast, it could be made more accessible by cleavage of the most proximal portion of the N-terminus. This 37 kD band does not appear to simply be a breakdown product of ETB receptor degradation, as intensity of this band was not increased in astrocytes treated with ET-1 and cycloheximide to down-regulate ETB receptors (Figure 3). It is unclear why the 37 kD band is absent from astrocyte-rich rat brain samples, but could be due to a difference in receptor processing between cells *in vivo* and those in culture. The 37 kD band may be a functional ETB receptor, as ETB receptors have been shown to maintain the ability to bind ET-1 despite N-terminal cleavage [25].

Our results show that some commonly used ETB receptor antibodies do not reliably detect ETB expression by Western blot and that ETB receptors can appear as either 37 kD or 50 kD bands. More research is needed to further characterize ETB receptor processing, but this study suggests that a 37 kD band can indicate the presence of functional ETB receptors. Detection of ETB receptors by Western blot is further complicated by differences in affinity for ETB-targeted antibodies for the 50 kD and 37 kD bands, thus the use of more than one antibody may be necessary to confirm ETB receptor expression depending on the form of ETB present and the primary antibody used.

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Figure 1.

Transfection of functional ETB receptors in HEK293 cells. **A.** qPCR for ETB receptors in native HEK293 (HEK) and HEK293 transfected with ETB receptors (HEK-ETB). ETB mRNA was barely detectable in HEK and was strongly increased, almost 800-fold, in transfected cells. Data is average $\pm$ SEM for $n = 3$ with expression normalized to cycloA and shown relative to HEK. **B.** Calcium imaging in HEK and HEK-ETB. HEK failed to show an increase in intracellular calcium in response to ET-1 (30 nM). In contrast, HEK-ETB showed a robust increase in intracellular calcium that was significantly reduced by the ETB receptor-selective antagonist BQ-788 (100 nM), but not the ETA receptor-selective antagonist BQ-123 (100nM). Antagonists were added 5 minutes before and then together with ET-1 (30 nM), data is average $\pm$ SEM for $n = 6$. **C.** Saturation binding of [^{125}I]-ET-1 in HEK and HEK-ETB. Transfection of ETB receptors dramatically increased binding of [^{125}I]-ET-1, with no signs of saturation at 300pM [^{125}I]-ET-1. **D.** Competition binding of BQ-788 with [^{125}I]-ET-1 in transfected cells. In HEK-ETB, BQ-788 competed with ET-1 binding in a monotonic fashion with an IC₅₀ of 188.6 nM.

Figure 2.

Primary antibodies were tested for their ability to detect endogenous ETB receptors in cultured rat astrocytes and rat brain homogenates, as well as transfected ETB receptors in HEK293 cells (HEK-ETB). Arrows show bands of interest that may represent ETB receptors. **A.** The IL2-targeted antibody labeled a prominent band near 50 kD that was present in rat brain, astrocyte and HEK-ETB, but not in HEK. **B.** The NT-targeted antibody labeled a band near 37 kD in astrocytes and HEK-ETB, but not in HEK or rat brain homogenate. **C.** and **D.** IL3- and CT-targeted antibodies both showed a prominent band near 60 kD in rat brain, astrocytes and HEK-ETB that was not present in HEK. A band could be detected near 50 kD in some ETB-expressing samples, but these bands were far less intense compared to the band near 60 kD. These antibodies also labeled many other, presumably non-specific bands that varied between samples, including a band near 85 kD in HEK.

Figure 3.

A. Representative Western blots from astrocyte samples treated with ET-1 (100 nM) and cycloheximide (20 μ g/mL) alone or in combination for 24 hours. Bands shown are the 50 kD, 37 kD and 60 kD bands labeled by the antibody listed in the left column along with corresponding GAPDH bands. As highlighted in Figure 2, these bands are the most likely to represent ETB receptors. **B.** Quantification of band intensity. Data are shown relative to control after normalization to GAPDH. 50 kD and 37 kD bands labeled by the IL2- and NT-targeted antibodies were significantly less intense in samples from cultures treated with ET-1/cycloheximide. In addition, treatment with ET-1 in the presence of cycloheximide caused a significant reduction in band intensity compared to cycloheximide alone. In

contrast, the 60 kD band labeled by the IL3-targeted antibody showed no difference in intensity across treatment groups and the 60 kD band labeled by the CT-targeted antibody was increased by ET-1/cycloheximide treatment. Bars are average $\pm$ SEM for n = 3. * Dunnett's test $p < 0.05$ compared to control, ^X t -test $p < 0.05$, ns = t -test not significant.

Table 1

ETB Receptor Antibodies

Source	Epitope	Species	[Primary]	[Secondary]	References
Abcam	2nd IL	Rabbit	1:500	1:1500	[14; 16; 27]
Santa Cruz	N-Terminus	Goat	1:500	1:1500	[5; 12; 28-29]
Alomone	3rd IL	Rabbit	1:500	1:1500	[2; 13; 23]
Abcam	C-Terminus	Rabbit	1:500	1:1500	