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Mechanisms of transcriptional activation of the mouse claudin-5 promoter by estrogen receptor alpha and beta

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ABSTRACT

Claudin-5 is an integral membrane protein and a critical component of endothelial tight junctions that control paracellular permeability. Claudin-5 is expressed at high levels in the brain vascular endothelium. Estrogens have multiple effects on vascular physiology and function. The biological actions of estrogens are mediated by two different estrogen receptor (ER) subtypes, ER alpha and ER beta. Estrogens have beneficial effects in several vascular disorders. Recently we have cloned and characterized a murine claudin-5 promoter and demonstrated 17beta-estradiol (E2)-mediated regulation of claudin-5 in brain and heart microvascular endothelium on promoter, mRNA and protein level. Sequence analysis revealed a putative estrogen response element (ERE) and a putative Sp1 transcription factor binding site in the claudin-5 promoter. The aim of the present study was to further characterize the estrogen-responsive elements of claudin-5 promoter. First, we introduced point mutations in ERE or Sp1 site in −500/+111 or in Sp1 site of −268/+111 claudin-5 promoter construct, respectively. Basal and E2-mediated transcriptional activation of mutated constructs was abrogated in the luciferase reporter gene assay. Next, we examined whether estrogen receptor subtypes bind to the claudin-5 promoter region. For this purpose we performed chromatin immunoprecipitation assays using anti-estrogen receptor antibodies and cellular lysates of E2-treated endothelial cells followed by quantitative PCR analysis. We show enrichment of claudin-5 promoter fragments containing the ERE- and Sp1-binding site in immunoprecipitates after E2 treatment. Finally, in a gel mobility shift assay, we demonstrated DNA–protein interaction of both ER subtypes at ERE. In summary, this study provides evidence that both a non-consensus ERE and a Sp1 site in the claudin-5 promoter are functional and necessary for the basal and E2-mediated activation of the promoter.

1. Introduction

The blood–brain (BBB) barrier plays a crucial role in the central nervous system (CNS) homeostasis. Changes of BBB integrity are common pathological features in different neuroinflammatory and neurodegenerative diseases of the CNS, so research that aims to clarify the molecular structure and molecular mechanisms of BBB regulation may open new avenues for developing future therapeutic strategies of CNS disorders. The BBB is built of endothelial cells sealed with tight junctions (TJ), which together with pericytes are surrounded by a basal lamina. Outside of the basal lamina astrocytic endfeet build a tight network having structural and regulatory functions (reviewed in (Abbott et al., 2010)). TJ are built through protein complexes composed of transmembrane, cytoplasmic and cytoskeletal proteins (Forster, 2008). Transmembrane proteins building the TJ are occludin, tricellulin and claudins. One of the prominently expressed claudins at the BBB is claudin-5 (Morita et al., 1999). Tight transcriptional and posttranslational regulation of claudin-5 takes place in physiological and pathological processes. Claudin-5 knockout mice show increased permeability of the BBB for molecules smaller than 800 Dalton and die several hours after birth (Nitta et al., 2003). Down-regulation of claudin-5 and elevation of BBB function were observed in a mouse model of multiple sclerosis (Errede et al., 2012), due to a HIV-1 infection (Andras et al., 2005); or at the hypoxic BBB as a result of stroke (Kleinschnitz et al., 2011). Over-expression of claudin-5 leads to an increase of barrier tightness (Ohtsuki et al., 2007) and has been demonstrated to have beneficial effects in the outcome of stroke or traumatic brain injury (Kleinschnitz et al., 2011; Thal et al., 2012).

Both subtypes of estrogen receptor, ERα and ERβ are expressed in the vasculature and in the CNS (Walter et al., 1985; Kuiper

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et al., 1996; Nilsson and Gustafsson, 2011). In the unliganded state, ERs are present in the cells in inactive form in the cytoplasm. After binding of the ligand, a steroid hormone 17β-estradiol (E2), ERs build homo- or heterodimers and translocalize into the nucleus, where they bind at the palindromic DNA sequences, so called estrogen response elements (EREs) in the promoter region of target genes leading to the activation of gene expression (Beato et al., 1995; Mangelsdorf et al., 1995). ER can also activate the transcription of genes binding to other transcription factors such as Sp1 or Ap1 (Kushner et al., 2000; Safe and Kim, 2004). Moreover, many different cofactors and inhibitors are involved in estrogen-mediated gene regulation (Shibata et al., 1997). Although both ERα and ERβ are expressed in endothelial and smooth muscle cells of blood vessels, most of E2 effects on the endothelium, such as re-endothelialization (Brouchet et al., 2001) and endothelial nitric oxide production (Darblade et al., 2002) are ERα-, but not ERβ mediated. In previous studies we cloned the murine claudin-5 promoter and characterized its estrogen-mediated regulation (Burek and Forster, 2009; Burek et al., 2010). E2 treatment of mouse brain and heart microvascular endothelial cells leads to an increase in transendothelial electrical resistance and in claudin-5 mRNA and protein level. These results were confirmed in ovariectomized mice and in ERβ-knockout mice giving a proof for ERβ-mediated regulation of claudin-5 in endothelial cells (Burek et al., 2010). Analysis of the claudin-5 promoter sequence (GenBank Accession number EU623469) revealed a non-consensus ERE at the position −303/−289 (5′-GGGTCATGCTGCTAA-3′) and a GC-rich sequence, a Sp1 binding site at the position −57/−47 (5′-TGGGGGCAGAG-3′) (Burek and Forster, 2009). So we raised the hypothesis that those sites might be responsible for E2-mediated regulation of claudin-5. The aim of the present study was to further characterize the molecular mechanism of E2-mediated regulation of claudin-5 and to identify the ER-binding sites in the claudin-5 promoter. For this we performed reporter gene assays using claudin-5 promoter with mutated potential ER binding sites, we analyzed binding of ER to the claudin-5 promoter chromatin immunoprecip itation assays (ChIP) using anti-ERα and ERβ antibody, and showed the complexes in elecritc mobility shift assay (EMSA). The results of this study suggest that both ER subtypes are involved in claudin-5 regulation cooperatively through both studied promoter elements, ERE and GC-rich sequence.

2. Materials and methods

2.1. Chemicals

17β-estradiol (E2) was purchased from Sigma. Stock solution was prepared in dimethyl sulfoxide (DMSO) and kept at −20 °C. Desired dilution was made shortly before the experiment in cell culture medium. The final concentration of DMSO in the treatment medium was lower than 0.01% (v/v).

2.2. Plasmids

Cloning of the mouse claudin-5 promoter (GenBank Accession number EU623469) into the pGL3-basic vector (Promega) was previously described (Burek and Forster, 2009). Constructs with −500/+111 and −268/+111 fragments of wild type claudin-5 promoter cloned upstream of luciferase reporter gene were used in this study.

2.3. Site-directed mutagenesis

Site-directed mutagenesis was performed using QuikChange II Site-Directed Mutagenesis Kit (Agilent) according to the manufacturer's instructions. The pGL3-basic −500/+111 and −268/+111 constructs containing the wild type claudin-5 promoter were used for generation of mutated forms. The primer (Eurofins MWG Operon) complementary to each other and containing the desired nucleotide changes were as follows: mutERE1: 5′-GTGTAGGTTAGGTTTCATGCTTCTAACAGTGGA-3′ and 5′-TCC ACT GTTAGAAGCA TGAAACCTAACCTACAC-3′; mutERE2: 5′-GTGTAGG TTAGGTTTCAT GCTG CTAA CAGTGGA-3′ and 5′-TCC ACT GTTAG-CAG CATGAAAC CTAACCTACAC-3′; mutSp1: 5′-TGCG CCC TGGTGA-CAGAGTCCG CCCCCGA-3′ and 5′-TCGGG GGCGG ACTC TG TCACCAGGGCGCA-3′. MutSp1 primer was used with both −500/+111 and −268/+111 constructs for a generation of a −500/+111 construct with a functional ERE and mutated Sp1 and for a generation of −268/+111 construct containing no ERE and a mutated form of Sp1 binding site. MutERE1 and mutERE2 were used to generate ERE mutations with 2- or 3-nucleotide changes. Plasmids were screened by restriction digestion and the mutations were confirmed by sequencing (Eurofins MWG Operon).

2.4. Cell culture

Mouse brain microvascular endothelial cells, cEND were isolated and immortalized as previously described (Forster et al., 2005; Burek et al., 2012). Cells were cultured in phenol-red free Dulbecco's modified Eagle's medium (DMEM) (Life Technologies, # 21063-029) with 10% fetal calf serum (FCS), and 100 U/ml penicillin–streptomycin on plates coated with collagen IV (Fluka) in a humid atmosphere with 5% CO_2 at 37 °C. Cells were allowed to reach confluence and were then transfected or treated with 10^{-8} M E2 in medium with 1% dextran-coated charcoal FCS as indicated in figure legends.

2.5. Transient transfection and reporter gene assay

cEND were seeded on 12-well cell culture plates (Greiner) coated with collagen IV and were grown for five days to confluence. Transient transfection was performed with Effectene reagent (Qiagen) according to the manufacturer's instructions in growth medium with 1% dextran-coated charcoal FCS. Promoter constructs cloned into the pGL3-basic vector (500 ng) and 250 ng of the internal control pTRL-TK encoding *Renilla* luciferase (Promega) for the estimation of the transfection efficiency were used. Cells were incubated for 24 h and then treated with 10^{-8} M E2 or vehicle for 24 h. The cells were lysed and harvested by the Dual-Luciferase reporter assay kit (Promega). Protein concentration was estimated using BCA Protein Assay Kit (Pierce). Luciferase activity was measured with LB 9507 luminometer with dual injector (Berthold Technologies). Each lysate was measured twice. Promoter activities were normalized for protein content and the activity of *Renilla* luciferase in each extract. The data were calculated as the mean of three or four identical set-ups.

2.6. Chromatin immunoprecipitation (ChIP) assay

Chromatin immunoprecipitation assays were carried out essentially as previously described (Harke et al., 2008) according to the Upstate Biotechnology protocol (Upstate). cEND cells (1×10^8) were grown in 10 cm tissue culture plates and were treated with a vehicle or with 10^{-8} M E2 for 24 h. Formaldehyde was added to the medium to a final concentration of 1%, and the cells were incubated for 10 min at 37 °C. Samples were lysed and sonicated three times for 45 s using a Bandelin Sonopuls Sonifier at 30% of maxi-

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