Molecular and Cellular Endocrinology 441 (2017) 108–115

Contents lists available at ScienceDirect

Molecular and Cellular Endocrinology

journal homepage: www.elsevier.com/locate/mce

Endothelial cells regulate β-catenin activity in adrenocortical cells *via* secretion of basic fibroblast growth factor

Carolin Schwafertz [a], Sven Schinner [a], Markus C. Kühn [a], Matthias Haase [a, b], Amelie Asmus [a], Birgit Mülders-Opgenoorth [a], Ishrath Ansurudeen [b, c], Peter J. Hornsby [d], Henning Morawietz [b], Elke Oetjen [e], Matthias Schott [a], Holger S. Willenberg [a, f, *]

[a] Division for Specific Endocrinology, Medical Faculty, Heinrich-Heine University Dusseldorf, D-40225 Dusseldorf, Germany
[b] Department of Medicine III, Carl Gustav Carus Medical School, University of Technology, D-01307 Dresden, Germany
[c] Department of Molecular Medicine and Surgery, L1:01 Rolf Luft Centrum, Karolinska Institute, Stockholm, Sweden
[d] Department of Physiology and Sam and Ann Barshop Institute for Longevity and Aging Studies, University of Texas Health Science Center, San Antonio, TX, USA
[e] Department of Clinical Pharmacology and Toxicology, Pharmacology for Pharmacist's Unit, University Medical Center Hamburg-Eppendorf, Hamburg, Germany
[f] Division of Endocrinology and Metabolism, Rostock University Medical Center, Germany

ARTICLE INFO

Article history:
Received 20 June 2016
Received in revised form
17 November 2016
Accepted 20 November 2016
Available online 23 November 2016

Keywords:
Aldosterone
Adrenal gland
Beta-catenin
Basic fibroblast growth factor
CITED2

ABSTRACT

Endothelial cell-derived products influence the synthesis of aldosterone and cortisol in human adrenocortical cells by modulating proteins such as steroidogenic acute-regulatory (StAR) protein, steroidogenic factor (SF)-1 and CITED2. However, the potential endothelial cell-derived factors that mediate this effect are still unknown. The current study was perfomed to look into the control of β-catenin activity by endothelial cell-derived factors and to identify a mechanism by which they affect β-catenin activity in adrenocortical NCI–H295R cells.

Using reporter gene assays and Western blotting, we found that endothelial cell-conditioned medium (ECCM) led to nuclear translocation of β-catenin and an increase in β-catenin-dependent transcription that could be blocked by U0126, an inhibitor of the mitogen-activated protein kinase pathway. Furthermore, we found that a receptor tyrosin kinase (RTK) was involved in ECCM-induced β-catenin-dependent transcription. Through selective inhibition of RTK using Su5402, it was shown that receptors responding to basic fibroblast growth factor (bFGF) mediate the action of ECCM.

Adrenocortical cells treated with bFGF showed a significant greater level of bFGF mRNA. In addition, HUVECs secrete bFGF in a density-dependent manner. In conclusion, the data suggest that endothelial cells regulate β-catenin activity in adrenocortical cells also *via* secretion of basic fibroblast growth factor.

1. Introduction

The adrenal gland is an effector organ of the hypothalamo-pituitary-adrenal axis and the renin-angiotensin system. As such, it is involved in the regulation of salt and water homeostasis, extracellular volume balance, blood pressure, immune function, and energy metabolism in response to stress.

The adrenal gland is heavily vascularized and almost every adrenocortical cell is in direct contact to an endothelial cell, whereby the microstructure is of relevance for proper adrenal function (Dobbie and Symington, 1966; Hornsby, 1987; Ehrhart-Bornstein et al., 1998). The regulation of adrenal blood flow is dependent on corticotropin (ACTH) (Vinson et al., 1985; Breslow, 1992, Thomas et al., 2003; Mohn et al., 2005; Zhang et al., 2007) and endothelial cell products affect the synthesis of adrenocortical hormones, including aldosterone (Vinson et al., 1985; Hinson et al., 1991; Nussdorfer et al., 1997; Ehrhart-Bornstein et al., 1998; Willenberg et al., 2008a,b).

Although a number of specific endothelial cell-derived factors have been identified, the overall effect of endothelial cell-derived products on adrenocortical cells has been poorly studied. Endothelial cell-conditioned medium (ECCM) is a cocktail of factors,

* Corresponding author. Division of Endocrinology and Metabolism, Rostock University Medical Center, Ernst-Heydemann-Str 6, D-18057 Rostock, Germany.
E-mail address: Holger.Willenberg@uni-rostock.de (H.S. Willenberg).

http://dx.doi.org/10.1016/j.mce.2016.11.015
0303-7207/

containing endothelin-1, angiotensin-2 (Kifor and Dzau, 1987; Vane and Botting, 1993), interleukin-6 (Willenberg et al., 2008a,b) and other, unidentified proteins (Ehrhart-Bornstein et al., 1998; Willenberg et al., 2008a,b). The aforementioned known components of the ECCM do not explain the effects observed when adrenocortical cells were incubated with ECCM (Ansurudeen et al., 2006, 2007; Willenberg et al., 2008a,b; Haase et al., 2009; Ansurudeen et al., 2009; Paramonova et al., 2010). However, Rosolowski et al. described an endothelium-derived 3000 Da protein which leads to increased aldosterone concentrations in supernatants of cultured bovine adrenal cells *via* the protein kinase C pathway (Rosolowsky and Campbell, 1994).

Interestingly, our studies have shown that the endothelial cells act on adrenocortical cells involving proteins essential for adrenocortical development, including steroidogenic factor (SF)-1, steroidogenic acute regulatory protein (StAR) and CITED2 (Ansurudeen et al., 2007; Haase et al., 2009). In addition, endothelial cell-derived products influence the proliferation of adrenocortical cells (Paramonova et al., 2010).

Recently, proliferation and tumorigenesis of adrenocortical adenomas were demonstrated to be associated with activation of β-catenin (Tissier et al., 2005; Stratakis, 2007, Schinner et al., 2009; Berthon et al., 2010; Gaujoux et al., 2011). Furthermore, knockdown of β-catenin leads to a decrease in SF-1 positive cells as well as to an early disappearance of adrenocortical and adrenomedullary precursor cells (Kim et al., 2008).

It was shown by other groups and ours that adrenocortical cells express frizzled receptors and β-catenin in the human adrenal gland and that activation of the Wnt signaling pathway stimulates aldosterone and cortisol biosynthesis through β-catenin (Chen and Hornsby, 2006, Schinner et al., 2007, 2009; Tadjine et al., 2008a,b; Schinner et al., 2009). We therefore hypothesized whether ECCM could regulate β-catenin-dependent transcription and if so whether the Wnt signaling pathway is involved in its regulation. We here show that ECCM significantly up-regulates β-catenin-dependent transcription through MAP- and RTK-pathways.

2. Materials and methods

This study aimed at better characterization of the ECCM effect on adrenocortical cells that was seen in previous studies whereby data on the adrenal hormonal responses and adrenal cell viability have been made available to the public (Ansurudeen et al., 2006, 2007, 2009; Haase et al., 2009; Paramonova et al., 2010). Further experiments were conducted as follows.

2.1. Cell cultures

For our *in vitro* studies, three cell lines were used. NCI–H295R cells were cultured in DMEM/F12-Glutamax medium (Invitrogen, Karlsruhe, Germany), containing fetal bovine serum (2%, Invitrogen), insulin (66 nM, Sigma-Aldrich, Munich, Germany), hydrocortisone (10 nM, Sigma-Aldrich), apo-transferrin (10 μg/mL, Sigma-Aldrich), β-estradiol (10 nM, Sigma-Aldrich), sodium selenite (30 nM, Sigma-Aldrich), penicillin (100 U/mL, Invitrogen) and streptomycin (100 mg/mL, Invitrogen), at 37 °C in a humidified atmosphere of 95% air, and 5% CO_2, as described previously (Ansurudeen et al., 2007).

Primary adrenocortical cells were established from an adrenal gland of a patient who had undergone unilateral nephrectomy for renal cancer. An immortalized as described previously (Suwa et al., 2001), using pLEGFP-CMV-SV40T and pBabe-hTERT retroviral vectors to produce immortalized adrenocortical cells. Immortalized adrenocortical (IMAC) cells were cultured in DMEM/F12-Glutamax (Invitrogen) with 10% fetal bovine serum (Invitrogen), penicillin (100 U/ml, Invitrogen) and streptomycin (100 mg/mL, Invitrogen) at 37 °C in a humidified atmosphere of 95% air, and 5% CO_2 for further experiments (Werminghaus et al., 2014).

At confluency, H295R and IMAC cells were subcultured using Accutase (PAA Laboratories, Cölbe, Germany) and seeded at density of 2×10^6 cells in 75-cm^2 flasks. For transfection and protein extraction, cells were plated in 24-well culture plates. At confluency of 80%, cells were used for experimental procedures.

Different preparations of human umbilical vein endothelial cells (HUVEC) were used and obtained from normal term and normal pregnancy donors. HUVECs have been isolated using the following protocol (Ansurudeen et al., 2007). After being emptied of blood by perfusion with phosphate buffer (50 mM, pH 7.0), 15-cm-long segments of umbilical cord venous or arterial vessels were filled with collagenase I (1 mg/mL of endothelial cell medium, PromoCell, Heidelberg, Germany) and incubated at 37 °C for 30 min. Cells were collected from the suspension by centrifugation at 50 *g* for 5 min at 4 °C. The cell pellet was suspended and cultured in endothelial cell basal medium (PromoCell), containing the ingredients of the supplement pack (PromoCell), fetal calf serum (10%) and antibiotics (penicillin 100 U/mL and streptomycin 100 μg/mL), at 37 °C in a humidified atmosphere of 95% air and 5% CO_2. For experiments, cells from passages 3–5 were used. Commercially available HUVEC and HUAEC preparations (Lonza, Walkersville, USA) were also used. At a confluency of over 80%, fresh DMEM/F12 glutamax medium with the supplemental pack was added to endothelial cells and incubated for 48–72 h to obtain ECCM. After this incubation period, ECCM was centrifuged to remove potential contaminat cells and stored at −20 °C until further use.

2.2. Plasmids and transfections

The plasmid TOPFLASH reporter gene (with multiple binding sites for TCF/LEF-transcription factors which are co-activated by β-catenin) and the FOPFLASH reporter gene (with mutated TCF/LEF-binding-sites) are commercially available at UPSTATE biotechnology (Lake Placid, USA). The plasmids pT81 for studies of cAMP-dependent protein kinase A transcription as well as the 1300 StAR-Luc plasmid were used as described previously (Oetjen et al., 1994; Caron et al., 1997). Cells were seeded at a density of 2×10^5 cells per well in 24-well culture plates. After 24 h, when confluency exceeded 50%, transfection was performed with Nanofectamin (PAA Laboratories, Cölbe, Germany) according to the manufacturer's protocol. The plasmids TOPFLASH, FOPFLASH, StAR-Luc and pT81 plasmids were transfected at a concentration of 0.5 μg per well along with the renilla luciferase pRLTK-Luc plasmid (Promega, Mannheim, Germany) at a concentration of 0.1 μg for internal control. 24 h later, the cells were stimulated with DMEM/F12 basal medium or ECCM, alongwith various inhibitors or proteins for next 24 h. The NCI–H295R cells and the immortalized cells were treated with different concentrations of ECCM diluted in DMEM/F12 (5%, 10%, 20%, 25%, 30%, 50%, 75%, and 100%). The effect of Wnt signaling in NCI–H295R cells was studied in the presence of inhibitors of the Wnt signaling pathway using sFRP-1 (secreted frizzled-related protein-1; 10 ng/mL, R&D Systems, Inc., Minneapolis, USA) which interacts with Wnt ligands and impedes their binding to one of the frizzled receptors, or Dkk-1 (Dickkopf-related protein 1; 1 μg/mL, R&D Systems, Inc.) which binds to the LRP5/6 co-receptor and inhibits signal transduction. The role of protein kinases in NCI–H295R cells were studied using inhibitors of protein kinase A (H89, 10 μM, Calbiochem Merck, Darmstadt, Germany), protein kinase B (Akt inhibitor VIII, 1 μM, Calbiochem Merck), protein kinase C (bisindolylmaleimide I, 3 μM, Calbiochem Merck), MEK inhibitor (U0126, 25 μM, Promega), PI3-kinase inhibitor (Ly294002, 50 μM, Sigma-Aldrich) and genistein (inhibitor of tyrosine-specific