Medizinische Fakultät der Charité - Universitätsmedizin Berlin
Campus Benjamin Franklin
aus dem Institut für Pharmakologie
Direktor: Univ. Prof. Dr. med. Walter Rosenthal

Ligand-induced N-terminal proteolysis of the human endothelin B receptor

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vorgelegt von
Evelina Grantcharova-Angelova
aus Sofia, Bulgarien

Referent: Priv.-Doz. Dr. med. Alexander Oksche

Korreferent: Priv.-Doz. Dr. med. H.-D. Orzechowski

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to my parents and my husband

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Abbreviations

AC	adenylyl cyclase
Ang II	angiotensin II
APS	ammonium persulfate
ATP	adenosine triphosphate
B_{max}	maximal binding
BSA	bovine serum albumin
cAMP	cyclic adenosine-monophosphate
CBB	Coomassie Brilliant blue G250
cDNA	complementary DNA
cpm	counts per minute
DMEM	Dulbecco's modified Eagle's medium
DNA	desoxyribonucleic acid
dpm	desintegration per minute
DSMZ	Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH
DTT	dithiothreitol
ECE-1	endothelin-converting-enzyme-1
EDTA	ethylendiamine-tetraacetate
EGF	epidermal growth factor
ER	endoplasmic reticulum
ERK1/2	extracellular-signal regulated kinase 1/2
pERK1/2	phosphorylated extracellular-signal regulated kinase 1/2
ET	endothelin
ET_B receptor	endothelin B receptor
FCS	fetal calf serum
FMP Berlin	Forschungsinstitut für Molekulare Pharmakologie, Berlin
GDP	guanosine diphosphate
GFP	green fluorescent protein
GPCR	G protein-coupled receptor

GRK	G protein-coupled receptor kinase
GTP	guanosine triphosphate
HEK 293 cells	human embryonic kidney 293 cells
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
IRL1038	ET_B receptor-selective antagonist
IRL1620	ET_B receptor-selective agonist
K_D	dissociation constant
K_i	inhibitory constant
LSM	Laser Scanning Microscopy
LT	low temperature
MAPK	mitogen activated protein kinase
MMP	matrix metalloprotease
NP-40	ethylphenyl-polyethylene glycol
OD	optical density
PAGE	polyacrylamide gel electrophoresis
PAR	protease-activated receptor
PBS	phosphate buffered saline
PGE1	prostaglandin E1
PKA	protein kinase A
PKC	protein kinase C
PLC	phospholipase C
PMSF	phenylmethylsulfonyl fluoride
PTX	pertussis-toxin
QM	quiescent medium
RES-701-1	ET_B receptor-selective antagonist
RNA	ribonucleic acid
rpm	rotations per minute
RT	room temperature
SDS	sodiumdodecyle sulfate
TACE	tumor necrose factor α-convertase
TBS	Tris-buffered saline
TEMED	N,N,N´,N´-Tetramethylethylene diamine

TM	transmembrane
Tris	Tris (hydroxymethyl)-aminomethan
Triton X-100®	Octylphenylpoly(ethylene glycol ether)$_n$
TSH receptor	thyrotrophic stimulating hormone receptor
V_2 receptor	vasopressin 2 receptor
VSMCs	vascular smooth muscle cells

5 Summary

The human endothelin B (ET_B) receptor comprises 442 amino acids, of which the first 26 function as a signal peptide. The signal peptide, which is essential for cell surface transport is cleaved off by a signal peptidase in the ER lumen during receptor biosynthesis. In addition, a second protheolytic cleavage within the extracellular N terminus (at R64↓S65) has been identified, which results in an N-terminally truncated receptor, lacking amino acids 26 to 64. The regulation and the physiological significance of this proteolysis were not known when this study was started. To gain more insight into the process of N-terminal proteolysis, ET_B receptor or ET_B·GFP fusion protein stably or transiently expressed in human embryonic kidney 293 (HEK 293) cells and in vascular smooth muscle cells (VSMCs) were analyzed. After incubation of cells with ^{125}I-ET-1 at 4°C, only the full-length ET_B receptor was detected at the cell surface. When cells were incubated at 37°C in the presence of endothelin-1, N-terminal cleavage was observed. The cleavage was not prevented by inhibitors of internalization (sucrose, phenylarsine oxide) or of serine and cysteinyl proteases. However, when cells were incubated with internalization and metalloprotease inhibitors (batimastat, inhibitor of TNFα-converting enzyme Ro32-7315) or metal chelators (EDTA, phenanthroline), the cleavage was blocked. The data show that metalloproteases mediate an agonist-dependent cleavage of the ET_B receptor at the cell surface.

Functional analysis of a mutant ET_B receptor lacking the first 64 amino acids (Δ2-64 ET_B) revealed normal ligand binding properties and preserved G protein-signaling (increase of inositol phosphate formation and inhibition of forskolin-induced cAMP-formation) when compared to the wild-type receptor. However, the Δ2-64 ET_B receptor showed a 15-fold reduced cell surface expression and an altered ability to activate ERK1/2. Although the wild-type and the Δ2-64 ET_B receptor elevated an early phase of ERK1/2 phosphorylation (within 5 min), only the wild-type receptor induced a second phase of ERK1/2 activation (starting after 80 min). The second phase was mediated *via* βγ subunit of G_i proteins and was abolished by inhibitors of matrix metalloproteases (batimastat and an inhibitor of TNFα-converting enzyme Ro32-7315).

The data presented in this study strongly suggest, that the N-terminal proteolysis of the human ET_B receptor is mediated by a metalloprotease in an agonist-dependent manner. Removal of the ET_B receptor's N terminus yields a receptor with a dramatically reduced cell surface expression and an altered ability to stimulate ERK1/2 activation. The data suggest, that the N-terminal cleavage of the ET_B receptor could be involved in the regulation of cell surface expression and of ERK1/2 activation. The functional role of the observed biphasic ERK1/2 activation *via* the full-length ET_B receptor and the monophasic ERK1/2 activation *via* the Δ2-64 ET_B receptor requires further characterization.