



Proapelin is processed extracellularly in a cell line-dependent manner with clear modulation by proprotein convertases

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Received: 26 June 2018 / Accepted: 28 October 2018 / Published online: 15 November 2018
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Abstract

Apelin is a peptide hormone that binds to a class A GPCR (the apelin receptor/APJ) to regulate various bodily systems. Upon signal peptide removal, the resulting 55-residue isoform, proapelin/apelin-55, can be further processed to 36-, 17-, or 13-residue isoforms with length-dependent pharmacological properties. Processing was initially proposed to occur intracellularly. However, detection of apelin-55 in extracellular fluids indicates that extracellular processing may also occur. To test for this, apelin-55 was applied exogenously to HEK293A cells overexpressing proprotein convertase subtilisin kexin 3 (PCSK3), the only apelin processing enzyme identified thus far, and to differentiated 3T3-L1 adipocytes, which endogenously express apelin, PCSK3 and other proprotein convertases. Analysis of culture media constituents from each cell type by high performance liquid chromatography–mass spectrometry and western blot demonstrated a time-dependent decrease in apelin-55 levels. This decrease was partially, but not fully, attenuated by PCSK inhibitor treatment in both cell lines. Comparison of the resulting apelin-55-derived peptide profile between the two cell lines demonstrated distinct processing patterns, with apelin-36 production apparent in 3T3-L1 adipocytes vs. detection of the prodomain of a shorter isoform (likely the apelin-13 prodomain, observed after additional proteolytic processing) in PCSK3-transfected HEK293A cells. Extracellular processing of apelin, with distinct cell type dependence, provides an alternative mechanism to regulate isoform-mediated physiological effects of apelin.

Keywords Apelin · Peptide hormone · Proprotein convertase subtilisin kexin · Proprotein processing · High performance liquid chromatography (HPLC) · Electrospray ionization time of flight mass spectrometry (ESI-MS)

Handling Editor: F. Albericio.

Electronic supplementary material The online version of this article (<https://doi.org/10.1007/s00726-018-2674-8>) contains supplementary material, which is available to authorized users.

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Abbreviations

AR	Apelin receptor (APJ)
BSA	Bovine serum albumin
DEXA	Dexamethasone
DMEM	Dulbecco's modified Eagle's medium
ESI-MS	Electrospray ionization mass spectrometry
FBS	Fetal bovine serum
GPCR	G protein-coupled receptor
HEK	Human embryonic kidney cells
His-apelin-55	Human apelin-55 with N-terminal His6 tag and TEV protease cleavage site
IBMX	3-isobutyl-1-methylxanthine
LC-MS	Liquid chromatography–mass spectrometry
NCS	New calf serum
P/S	Penicillin and streptomycin
PCSK	Proprotein convertase subtilisin kexin (PCSK)
PRCP	Prolylcarboxypeptidase
Pyr	Pyroglutamate

RP-HPLC	Reverse phase high performance liquid chromatography
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEM	Standard error of the mean
TFA	Trifluoroacetic acid
TBST	Tris-buffered saline with 0.1% Tween-20

Introduction

Apelin is a cognate peptide hormone for the class A G-protein coupled receptor (GPCR) called the apelin receptor (AR, formerly APJ). Both apelin and the AR are widely expressed in the body, including in the central nervous system, cardiovascular system, and other peripheral locations such as adipose and muscle tissues (Shin et al. 2018). Consequently, apelin is known to regulate various physiological systems. Its effects include modulation of heart contractility, vasodilation, and glucose homeostasis. In turn, its potential for therapeutic application means that apelin has been a highly sought after medicinal chemistry target (Narayanan et al. 2015).

Cleavage of a 22-residue signal peptide from the 77-residue human apelin expression product (typically termed proapelin) produces apelin-55 (Table 1). Apelin-55 has traditionally been referred to as proapelin, although this nomenclature is not consistent with its recently demonstrated (Shin et al. 2017a) bioactivity. The apelin-55 sequence contains multiple dibasic amino acid motifs (Tatemoto et al. 1998), indicative of the potential involvement of proprotein convertase subtilisin kexin (PCSK)-mediated processing (Seidah and Prat 2012) to produce 36-, 17-, and 13-residue isoforms (Table 1). All isoforms include the C-terminal residues of their shared unprocessed protein, required for receptor binding and activation (Shin et al. 2018).

In support of PCSK involvement in apelin processing, we previously demonstrated that apelin-55 can be processed by PCSK3 (furin) in vitro (Shin et al. 2013). Notably,

PCSK3-mediated processing of apelin-55 was direct and preferential, with only apelin-13 produced. Despite similar dibasic site cleavage motifs, no additional longer apelin isoforms could be detected by liquid chromatography–mass spectrometry (LC-MS), and neither PCSK1 (PC1/3) nor PCSK7 (PC7) could process apelin-55 in vitro. Apelin-36 has since also been shown to be processed by PCSK3 (Adam et al. 2016).

Although their conformations are highly similar in solution, indicative of similar modes of AR activation (Shin et al. 2017a), apelin isoforms present length-dependent variation in receptor affinity, signaling, and cellular effects (Tatemoto et al. 1998; Habata et al. 1999; Shin et al. 2017a). For example, apelin-13 rapidly dissociates from the receptor upon internalization, leading to receptor recycling (Evans et al. 2001; Lee et al. 2010). In contrast, apelin-36-AR interaction leads to receptor degradation with the ligand and receptor remaining associated upon internalization (Lee et al. 2010). This phenomenon is consistent with the higher receptor affinity reported for the longer isoform (Habata et al. 1999). These disparities mean that the ratio of apelin isoforms present in a given setting has been hypothesized to regulate the physiological effects of apelin (Lee et al. 2010).

Apelin-55 was not detected when apelin was first identified in bovine stomach tissue extract (Tatemoto et al. 1998). Instead, apelin-36 was the longest isoform identified. Consequently, apelin-55 was labeled as an inactive proprotein and apelin-77 as its preproprotein. These observations, collectively, led to the hypothesis that apelin processing occurred intracellularly, with apelin-36 produced from the 55-residue proprotein isoform, followed by subsequent processing to the shorter and more potent apelin-13 or -17 isoforms (Kleinz and Davenport 2005; Habata et al. 1999). In support of intracellular processing, co-expression of apelin and PCSK3 led to observable cleavage of apelin into shorter isoforms (Adam et al. 2016). However, the detection of intact apelin-55 in colostrum and milk (Mesmin et al. 2011) and of isoforms with mass greater than apelin-36 in plasma (Foldes et al. 2003) suggest that intracellular processing to apelin-36 is

Table 1 Apelin isoform amino acid sequences

Identity	Amino acid sequence*
Apelin-77 (Preproapelin)	<u>M</u> NLRLCVQALLLLWLSLTAVCGGSLMPLPDGNGLEDGNNRHLVQPRGSRNGPGPWQGGRRKFRRQRPRLSHKGMPMF
Apelin-55 (Proapelin)	GSLMPLPDGNGLEDGNNRHLVQPRGSRNGPGPWQGGRRKFRRQRPRLSHKGMPMF
Apelin-36	LVQPRGSRNGPGPWQGGRRKFRRQRPRLSHKGMPMF
Apelin-17	KFRRQRPRLSHKGMPMF
Apelin-13	QRPRLSHKGMPMF
Pyr-apelin-13	<ERPRLSHKGMPMF

*Underlined residues in preproapelin represent the signal peptide; <E represents the N-terminal pyroglutamate that is spontaneously formed from glutamine

not ubiquitous. Correspondingly, we recently demonstrated that apelin-55 activates the AR (Shin et al. 2017a).

Secretion of intact apelin-55 means that this isoform may directly interact with endoproteases on the cell surface or another extracellular context to undergo enzymatic processing. In support of this, while apelin-36 was detected in the mixture of bioactive peptides purified from bovine stomach tissues (Tatemoto et al. 1998), only Pyr-apelin-13 (apelin-13 with an N-terminal pyroglutamate) was detectable from glucose-stimulated gastric secretion in mice (Dray et al. 2013). Thus, if apelin-36 is the form released from stomach cells, the detection of differing isoforms in gastric secretions implies that processing may occur extracellularly. Furthermore, while injection of apelin-36 or -13 resulted in an antithrombotic response, an apelin-36 mutant incapable of being processed by PCSKs elicited no response, indicating that apelin-36 must be processed to the 13-residue form upon injection for this activity (Adam et al. 2016).

Although there are likely multiple proteases capable of processing apelin to shorter bioactive forms, as distinct from proteases known to produce inactive or less active forms (Vickers et al. 2002; McKinnie et al. 2016; Wang et al. 2016; Kehoe et al. 2016), only PCSK3 has, thus far, been demonstrated to process apelin (Shin et al. 2013; Adam et al. 2016). This proprotein convertase is expressed ubiquitously (Seidah 2011; Thomas 2002). It can be membrane-anchored through a transmembrane domain, either in the secretory pathway or on the cell surface (Teuchert et al. 1999; Mayer et al. 2003, 2004). Alternatively, it may be shed into the extracellular fluid upon transmembrane domain cleavage (Vidricaire et al. 1993; Molloy et al. 1999). Either the membrane-anchored or shed forms of PCSK3 would potentially allow for extracellular processing. This follows precedents of extracellular processing of anthrax toxin (Klimpel et al. 1992), human immunodeficiency virus type I protein Vpr (Xiao et al. 2008), pro-ADAMTS9 (Koo et al. 2006), and B-type natriuretic peptide (Semenov et al. 2010). Notably, various apelin isoforms also bind to membrane-mimetic micelles (Langelaan and Rainey 2009; Shin et al. 2017b), further increasing the likelihood of apelin-PCSK3 interactions on the cell surface through the combination of factors proposed to increase favorability of peptide-receptor interactions in the “membrane catalysis” mechanism (Sargent and Schwyzer 1986).

All of these factors imply strong potential for extracellular apelin processing by cell surface enzymes such as PCSK3. To test for this, we exposed exogenous hexahistidine (His₆)-tagged apelin-55 to PCSK3-overexpressing human embryonic kidney (HEK293A) cells, providing a situation with a high probability of observing extracellular processing. Apelin processing was also tested upon exposure to differentiated 3T3-L1 adipocytes, which express both apelin and PCSK3 endogenously (Shin et al. 2013) and which

have been extensively used to study the role of apelin in adipoinular axis at both cellular and molecular level (Yue et al. 2011; Than et al. 2012, 2015; Masaki et al. 2012). The culture medium from each cell line was characterized through reverse phase high performance LC (RP-HPLC), electrospray ionization MS (ESI-MS), and western blotting to determine both the extent and isoform specificity of extracellular apelin processing taking place in each context.

Materials and methods

His-apelin-55 production and purification

Human apelin-55 with an N-terminal His₆ tag and TEV protease cleavage site (His-apelin-55; total 75 residues) was expressed in *Escherichia coli* C41(DE3) and purified using Ni-NTA affinity and cation exchange chromatography, employing minor modifications to previous protocols (Shin et al. 2013). Briefly, the protein was purified by cation exchange chromatography without TEV protease cleavage to provide an N-terminally His₆-tagged apelin-55 amenable to downstream antibody-based detection. The His₆-tagged peptide was further purified using RP-HPLC, as detailed previously (Shin et al. 2017a), using a ProStar HPLC (Varian Canada Inc.) equipped with a preparative C₁₈ column (20 mm I.D. × 250 mm, Cosmosil, Nacalai USA Inc.), with a binary solvent system (A: H₂O with 0.1% trifluoroacetic acid (TFA) and B: acetonitrile with 0.1% TFA; flow rate 8 mL/min). Gradients were as follows: (i) 2–20% solvent B in 5 min, (ii) 20–45% solvent B in 25 min, (iii) 45–2% solvent B in 1 min. Eluent peaks were collected, lyophilized, and analyzed by ESI-MS (Mass Spectrometry Laboratory, Dalhousie University). Lyophilized, purified His-apelin-55 aliquots were prepared based upon the Beer Lambert law ($c = A \cdot \epsilon^{-1} \cdot l^{-1}$, where A is absorbance at a given wavelength (280 nm), $\epsilon_{280 \text{ nm}} = 6990 \text{ M}^{-1} \text{ cm}^{-1}$ is the molar absorptivity at 280 nm for the fusion protein calculated as described by Gill and von Hippel (Gill and von Hippel 1989), and l is the pathlength).

Cell culture

HEK293A cells (ATCC) were cultured at 37 °C in 5% CO₂ in Dulbecco's modified Eagle's medium (DMEM, Sigma-Aldrich) containing 10% fetal bovine serum (FBS, Invitrogen, USA), 1% penicillin and streptomycin (P/S, P4333, Sigma-Aldrich). 3T3-L1 preadipocytes (ATCC) were cultured at 37 °C in 5% CO₂ in high glucose DMEM containing 10% new calf serum (NCS, Invitrogen) and 1% P/S. The medium for both cell lines was refreshed every second day, with transfer accomplished by 10% trypsin digest when cells were at ~70% confluence.

HEK293A culture and transfection

HEK293A cells were seeded in 12-well plates and cultured in high glucose DMEM containing 1% P/S and 10% FBS. At ~80% confluency, they were transfected by addition of serum-free 625 μL of Opti-MEM (High Glucose, Gibco) containing 1 μg of the PCSK3 coding pIRES2-EGFP plasmid (gift of Drs. Michel Chrétien and Janice Mayne, University of Ottawa) and 2.5 μL of Lipofectamine 2000 (Invitrogen) per well. Exposure to transfection medium was carried out for 5 h, then medium was replaced with high glucose DMEM containing 10% FBS. Transfected cells were allowed to express the PCSK3 gene for 48 h prior to use in the extracellular processing assay. To check the efficiency of transfection, total RNA was extracted from HEK293A cells using Aurum™ Total RNA Fatty and Fibrous Tissue Kit (Bio-Rad) according to the manufacturer's protocol. Total RNA was reverse transcribed using the iScript™ cDNA Synthesis Kit (Bio-Rad) and PCSK3 mRNA was amplified by polymerase chain reaction (primers - forward: 5'-AACAAACGGTGTCTGTGGTGTAGGT-3'; reverse: 5'-AGCTGGCACTGTAGATGTGGATGT-3').

3T3-L1 preadipocyte differentiation

3T3-L1 preadipocytes were seeded in 12-well plates and cultured to confluence. At 2 days post-confluence (day 0), the medium was changed to induction medium (DMEM, 10% FBS, 1% P/S, 1 μM dexamethasone (DEXA, D2915, Sigma-Aldrich), 0.5 mM 3-isobutyl-1-methylxanthine (IBMX, I7018, Sigma-Aldrich), and 1 $\mu\text{g}/\text{ml}$ human insulin (HI-210, Eli Lilly and Company). After 2 days of incubation (day 2), the medium was replaced with insulin medium (DMEM supplemented with 10% FBS, 1% P/S, and 10 $\mu\text{g}/\text{ml}$ human insulin). Thereafter, the medium was replaced every day with FBS medium (DMEM supplemented with 10% FBS and 1% P/S). Once full differentiation was observed (day 8–10), adipocytes were used for extracellular apelin-55 processing assays.

Extracellular apelin-55 processing assay

Following the final step of cell setup (HEK293A transfection or 3T3-L1 differentiation), His-apelin-55 was dissolved (3 $\mu\text{g}/\mu\text{L}$; 358 μM) in phenol red-free DMEM (High Glucose, Sigma-Aldrich). Medium was replaced with serum-free and phenol red-free DMEM (High Glucose, 400 μL /well). Each well of the plate was supplemented with dissolved protein (50 μL) at a final concentration of 0.333 $\mu\text{g}/\mu\text{L}$ per well. Cells were exposed to His-apelin-55 for designated time points. For inhibition studies, cells were exposed to decanoyl-RVKR-CMK (Biomol International) in serum-free and phenol red-free DMEM (High Glucose, 400 μL /

well) at concentrations of 25, 2.5, or 0.25 μM for 1 h prior to supplementation with His-apelin-55. For 0 h (control) samples, the culture medium was collected promptly upon addition of His-apelin-55; thus, exposure to His-apelin-55 was short ($\leq \sim 1$ min). For all other treatments, the culture medium was collected at the designated incubation periods. All treatments were carried out in triplicate and all experiments were at least in duplicate. A high inhibitor treatment (25 μM) was used as a control for all inhibitor experiments.

RP-HPLC-MS based detection

Culture media samples were resolved using a ProStar HPLC employing a C_{18} analytical column (4.6 mm I.D. \times 150 mm, Grace Alltech) and a binary solvent system (A: H_2O with 0.1% trifluoroacetic acid (TFA) and B: acetonitrile with 0.1% TFA; flow rate 1 mL/min) with gradient elution as follows: (i) 2–20% solvent B in 5 min, (ii) 20–40% solvent B in 20 min, (iii) 40–100% solvent B in 15 min, (iv) 100–2% solvent B in 1 min. UV chromatograms were recorded at 213 and 280 nm simultaneously. Non-culture media eluents were collected and pooled for each experiment then lyophilized. Eluent masses were then determined using ESI-MS (Mass Spectrometry Laboratory, Dalhousie University; instrument background shown for reference in Fig. S1) and compared to all masses that could theoretically result from post-translational processing of His-apelin-55 for identification.

Western blotting-mediated detection

For detection of extracellular His-apelin-55, 30 μL of collected culture media from each well (~ 10 μg protein load, based on initial His-apelin-55 concentration prior to cell exposure) were resolved by SDS-PAGE (20% polyacrylamide gel, 120 V, 2 h) for each experiment. The resulting gel was transferred to a polyvinylidene difluoride (PVDF) membrane (Bio-Rad) at 100 V for 45 min. Membranes were blocked in Tris-buffered saline with 0.1% Tween-20 (TBST) containing 25 mg/ml BSA for 60 min at room temperature and then washed twice with TBST for 10 min each before incubation with HisProbe-HRP (Thermo Fisher Scientific; 1:5000 dilution in TBST with 25 mg/ml BSA) for 60 min. Subsequently, membranes were washed with TBST four times for 5 min each then incubated in Clarity Western ECL Blotting Substrates (Bio-Rad). Chemiluminescence was detected and His-apelin-55 band density was quantified using a molecular imager (Gel Doc™ XR+, Bio-Rad). Each quantified value was standardized to the intensity of the unprocessed His-apelin-55 control lane (i.e., 0 h) for the respective membrane blot. The standardized values from all experiments were then combined for statistical analyses. Results are presented as mean \pm standard error of the mean (SEM) and were analyzed using the *t* test to compare each

inhibitor treatment to the control at 0 h (a significance of $P < 0.05$ was applied).

Results

His-apelin-55 is processed upon extracellular introduction to HEK293A cells

To evaluate potential products arising from exogenous His-apelin-55 [mass (Table 2) confirmed by ESI-MS (Fig. S2)] processing, culture media constituents were resolved by RP-HPLC. The level of intact His-apelin-55 was significantly decreased upon incubation in medium with HEK293A cells as a function of the duration of exposure (Fig. 1 and Fig. S3). Processing to specific isoforms was also implied, given the appearance of multiple new eluent peaks from ~15 to 20 min corresponding to RP-HPLC water:acetonitrile proportions previously observed for apelin isoform elution (Shin et al. 2013). These changes were dependent on cell exposure, as level of His-apelin-55 remained stable in cell-free culture medium (Fig. S4), consistent with our previous findings on apelin-55 stability in various buffer-based experimental conditions (Shin et al. 2013; Shin et al. 2017a).

Increased PCSK3 levels in HEK293A cells by transient transfection (Fig. S5) also decreased the His-apelin-55 level, without affecting the processing pattern in comparison to non-transfected cells (Fig. 1). Despite this decrease in His-apelin-55 level, the eluent peaks resulting from processing were not observed to correspondingly increase. To maximize the yield of extracellular apelin processing under these experimental conditions, PCSK3-transfected HEK293A cells were used for subsequent experiments.

ESI-MS analysis was carried out for the eluent peaks collected during RP-HPLC separation (masses of all potential processing products considered are detailed in Table S1). The second largest peak at an elution time of ~17 min had a mass corresponding to His-apelin-55 Δ 15 (Table 2, Fig. S6). The eluent at ~17.5 min corresponded to a peptide with a mass consistent with His-apelin-55 Δ 32 (Table 2, Fig.

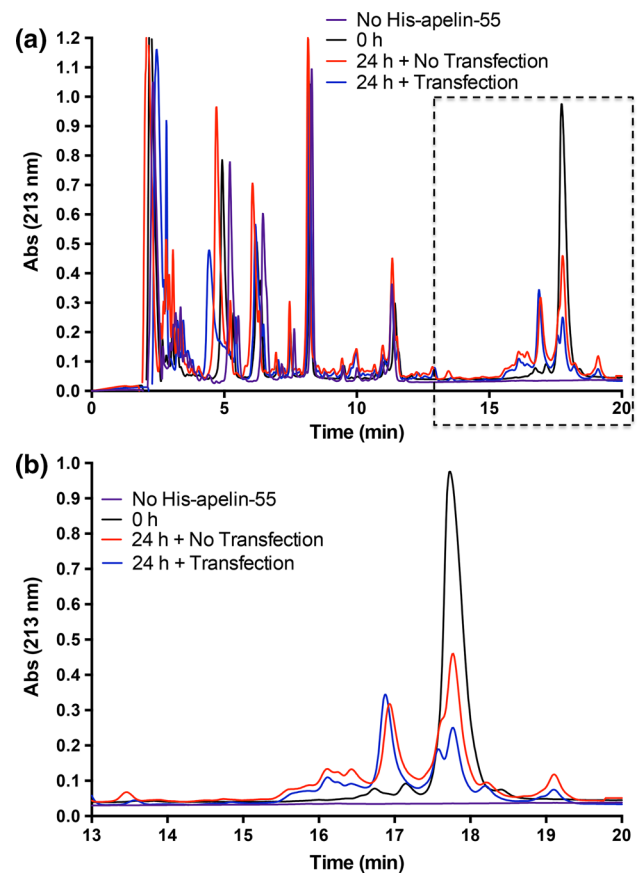


Fig. 1 HEK293A cells process exogenous His-apelin-55 (eluent peak at 18 min) in a PCSK3-dependent manner. **a**, **b** Representative RP-HPLC chromatograms of cell culture media supplemented with exogenous His-apelin-55. After incubation with HEK293A cells, the resulting supernatant was resolved using a C_{18} analytical RP-HPLC column (linear gradient from 2 to 45% acetonitrile in 0.1% (v/v) aqueous TFA). **b** Expansion of the 13–20 min RP-HPLC chromatograms from panel **a** (indicated by dashed box)

S7 and Fig. S8). Eluents over the 15–16.5 min exhibited convoluted m/z ratios corresponding to various processed products (Table S1, Fig. S9 and Fig. S10). Similar to the eluent at 17 min, His-apelin-55 cleavage products consistent with many of these corresponded to loss of 15 C-terminal

Table 2 Nomenclature, mass, and amino acid sequence of major exogenous His-apelin-55 processing products observed after incubation in HEK293A cell or 3T3-L1 adipocyte culture medium

Name	Mass (Da)	Amino acid sequence*
His-apelin-55	8380	<u>SGSHHHHHHGSSGENLYFQ</u> SGSLMPLPDGNGLDGNVRLVQPRGSRNGPGPWQGGRRK-FRRQRPRLSHKGPMPF
His-apelin-55 Δ 1	8233	<u>SGSHHHHHHGSSGENLYFQ</u> SGSLMPLPDGNGLDGNVRLVQPRGSRNGPGPWQGGRRK-FRRQRPRLSHKGPMP
His-apelin-55 Δ 15	6335	<u>SGSHHHHHHGSSGENLYFQ</u> SGSLMPLPDGNGLDGNVRLVQPRGSRNGPGPWQGGRRKF
His-apelin-55 Δ 32	4202	<u>SGSHHHHHHGSSGENLYFQ</u> SGSLMPLPDGNGLDGNVRLVQP
His-apelin-55 Δ 36	4196	<u>SGSHHHHHHGSSGENLYFQ</u> SGSLMPLPDGNGLDGNVRLH

*Underlined residues represent N-terminal His₆ tag and TEV protease cleavage site

residues, but with additional N- and, in some cases, up to 16 C-terminal truncations (Table S1).

PCSK inhibitor decreases the quantity of processing

To further test the putative role of PCSK3 in the observed extracellular processing of His-apelin-55, the inhibitor decanoyl-RVKR-CMK was employed. This compound is a general PCSK inhibitor and has been previously shown to effectively inhibit processing activity of various PCSK subtypes, including PCSK3, both intracellularly and at the cell surface (Koo et al. 2006; Remacle et al. 2010). Addition of decanoyl-RVKR-CMK to the culture medium of PCSK3-overexpressing HEK293A cells decreased exogenous His-apelin-55 processing, as was evident by HPLC elution and western blotting profiles (Figs. 2, 3a). Densitometry demonstrated statistically significant inhibition with both 25

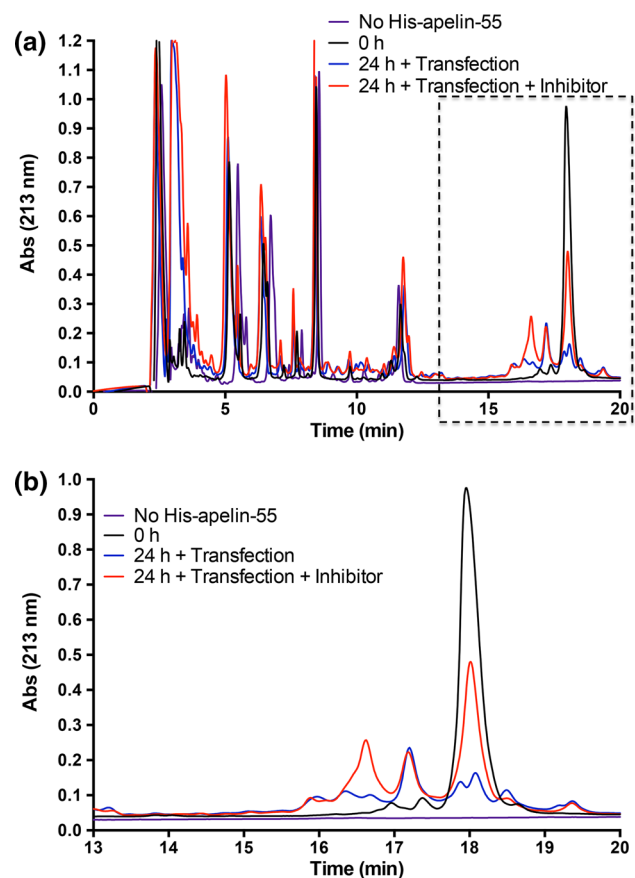


Fig. 2 The PCSK inhibitor decanoyl-RVKR-CMK alters exogenous His-apelin-55 processing by HEK293A cells overexpressing PCSK3. **a**, **b** Representative RP-HPLC chromatograms of culture media of cells pretreated with the inhibitor for 1 h prior to supplementing with exogenous His-apelin-55. The resulting supernatant was resolved using a C₁₈ analytical RP-HPLC column (linear gradient from 2 to 45% acetonitrile in 0.1% (v/v) aqueous TFA). **b** Expansion of the 13–20 min RP-HPLC chromatograms from panel **a** (indicated by dashed box)

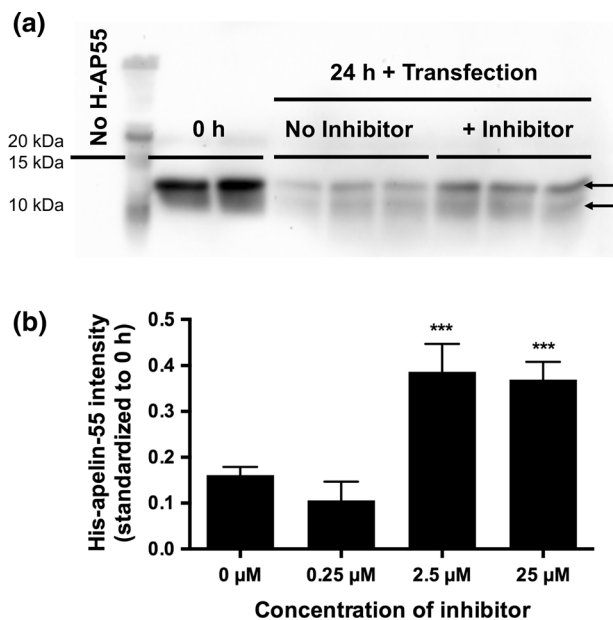


Fig. 3 Decanoyl-RVKR-CMK inhibits exogenous His-apelin-55 processing by HEK293A cells overexpressing PCSK3. Cells were pretreated with the indicated concentrations of PCSK inhibitor for 1 h prior to supplementing culture media with exogenous His-apelin-55. **a** Representative western blot. After the indicated incubation time, culture media were collected and a 30 μL sample of the medium from each well (~10 μg protein load, based on His-apelin-55 concentration prior to cell exposure) was resolved by SDS-PAGE and transferred onto PVDF membrane for blotting ($n=3$ wells, $N\geq 2$ experiments). Arrows denote the unprocessed (upper) and processed (lower) His-apelin peptides. **b** Densitometry-based intensities of His-apelin-55 bands normalized to the respective 0 h control. Results are mean \pm SEM of the normalized values. Statistical significance (t test; *** $P < 0.001$) of each PCSK inhibitor dose was tested with respect to the 0 μM control

and 2.5 μM inhibitor doses (Fig. 3b); however, pretreatment with the inhibitor even at the higher of these doses did not completely block processing. Namely, despite a single apparent peak at ~19 min, a species consistent with His-apelin-55 Δ 32 remained detectable by ESI-MS (Table 2, Fig. S11), implying that this processing product is still present, though not observed through UV absorption-based monitoring of HPLC elution. Notably, HPLC chromatograms also exhibited a prominent elution peak at ~16 min with the addition of inhibitor (Fig. 2) with a mass consistent with His-apelin-55 Δ 1 (Table 2, Fig. S12; Table S2 provides all masses considered).

His-apelin-55 is processed by 3T3-L1 adipocytes

Exogenous His-apelin-55 processing was also examined in the presence of 3T3-L1 adipocytes. Given that mRNA expression of PCSK1, 3, and 7 increase in 3T3-L1 cell line upon differentiation (Shin et al. 2013), fully differentiated 3T3-L1 adipocytes were tested for extracellular apelin-55

processing due to the correspondingly increased likelihood of PCSK-mediated processing. Similarly to incubation with HEK293A cells, incubation with differentiated adipocytes decreased the level of exogenous His-apelin-55 following 24 h (Figs. 4, 5 and Fig. S13).

HPLC analysis of adipocyte culture medium exhibited striking differences relative to HEK293A culture medium. Specifically, the elution profile of His-apelin-55 and its processed products widened to a range of ~13–20 min (Fig. 4), the overall chromatographic resolution was lowered, and the number of distinguishable peaks decreased. Analysis of the resulting eluents by ESI-MS also demonstrated distinct products from those observed in HEK293A culture medium (Table S3 details all processing products and masses considered for adipocyte culture medium). In particular, the product eluting at ~16 min exhibited a mass corresponding to His-apelin-55 Δ 36 (Table 2, Fig. S14). Consistent with this,

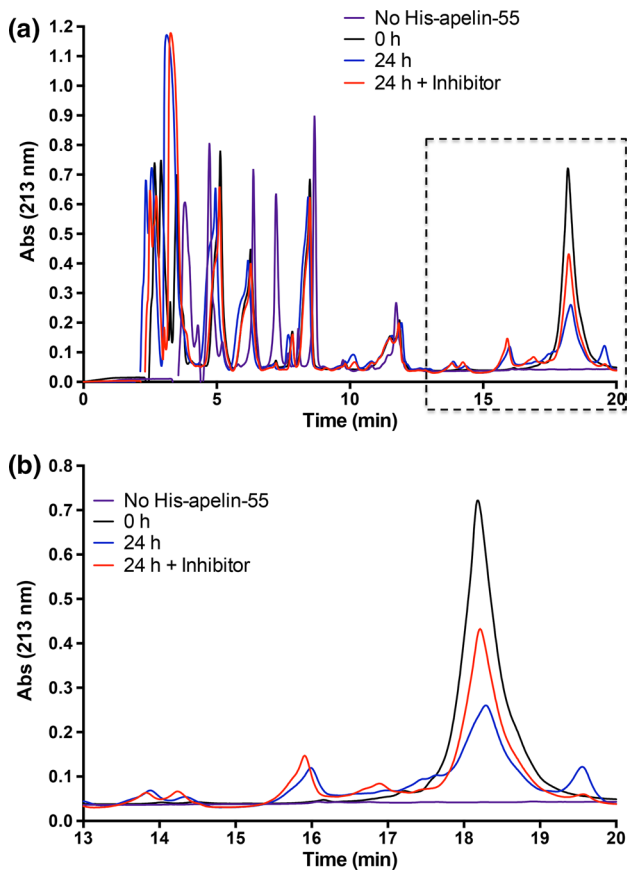


Fig. 4 Exogenous His-apelin-55 is processed by 3T3-L1 adipocytes and can be inhibited by decanoyl RVKR-CMK. **a**, **b** Representative RP-HPLC chromatograms. Cells were pretreated with the inhibitor for 1 h prior to supplementing culture media with exogenous His-apelin-55. The resulting supernatant was resolved using a C₁₈ analytical RP-HPLC column (linear gradient from 2 to 45% acetonitrile in 0.1% (v/v) aqueous TFA). **b** Expansion of the 13–20 min RP-HPLC chromatograms from panel **a** (indicated by dashed box)

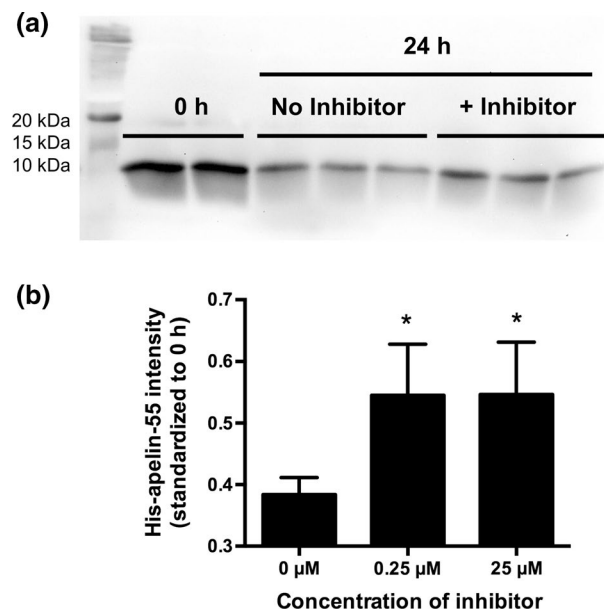


Fig. 5 Decanoyl-RVKR-CMK inhibits exogenous His-apelin-55 processing by 3T3-L1 adipocytes. Cells were incubated with the indicated concentrations of PCSK inhibitor for 1 h prior to supplementing culture medium with exogenous His-apelin-55. **a** Representative western blot. After indicated incubation time, cultured media were collected and a 30 μ L sample of the medium from each well (~10 μ g protein load, based on His-apelin-55 concentration prior to cell exposure) was resolved by SDS-PAGE and transferred onto PVDF membrane for blotting ($n=3$ wells, $N\geq 2$ experiments). **b** Densitometry-based intensities of His-apelin-55 bands normalized to the respective 0 h control. Results are mean \pm SEM of the normalized values. Statistical significance (t test; *** $P<0.001$) of each PCSK inhibitor dose was tested with respect to the 0 μ M control

the eluents from 13 to 15 min exhibited masses matching apelin-31 and -36, respectively (Fig. S15).

Pretreatment of 3T3-L1 adipocytes with decanoyl-RVKR-CMK again reduced, but did not completely block, His-apelin-55 processing (Fig. 5). In contrast to HEK293A culture medium, where a minimum inhibitor dose of 2.5 μ M was required, a dose of 0.25 μ M effectively inhibited His-apelin-55 processing in 3T3-L1 culture medium (Fig. 5b). Also distinct from HEK293A cells (Fig. 3a), lower molecular weight bands indicative of new His₆-reactive processing products were not observed by western blotting in adipocyte culture medium (Fig. 5a).

Discussion

The potential for extracellular processing of apelin in vivo is plausible when coupling: (1) the extracellular detection of all canonical bioactive apelin isoforms (Table 1) including apelin-55 (Foldes et al. 2003; De Mota et al. 2004; Miettinen et al. 2007; Azizi et al. 2008; Mesmin et al. 2011; Zhen et al. 2013) with (2) the potential for PCSKs to be

cell surface-anchored and/or circulating (Seidah 2011) and (3) PCSK-mediated processing of exogenous proteins (Xiao et al. 2008; Semenov et al. 2010; Klimpel et al. 1992). Processing in this manner would alter the balance of isoforms in proximity to the receptor. Given isoform-dependent pharmacology (Tatemoto et al. 1998; Shin et al. 2017a; Lee et al. 2010), this would, in turn, affect downstream signaling.

When incubated with HEK293A cells, exogenous His-apelin-55 was processed to a number of shorter forms (blue arrows, Fig. 6). This exhibited a PCSK3-dependence, as processing was increased with PCSK3 transfection and overexpression and was attenuated by PCSK inhibitor treatment. Based upon our *in vitro* observation of specific apelin-13 production from apelin-55 by PCSK3 (Shin et al. 2013), it is likely that apelin-13 is produced and the resulting His-apelin-55 Δ 13 N-terminal domain is further processed by Arg exoproteases, such as carboxypeptidase M that is found on the surface of kidney cells (Skidgel et al. 1989). This would, in turn, produce the major observed product, His-apelin-55 Δ 15. Consistent with a steady-state level of this truncated N-terminal domain production, the quantity of His-apelin-55 Δ 15 did not significantly change upon pretreatment with either PCSK3 overexpression or PCSK inhibitor treatment.

Apelin-13 is also known to have a relatively short half-life (< 1 h) in biological fluids such as plasma (Murza et al. 2012, 2014), with a variety of medicinal chemistry-oriented research efforts specifically targeted at improving its stability (Narayanan et al. 2015). This is consistent with our inability to specifically isolate and observe apelin-13 from culture medium through HPLC purification. Although it would certainly have been ideal to observe this isoform to unambiguously demonstrate this as the C-terminal cleavage product, the most likely source of His-apelin-55 Δ 15 is its production as the cleaved N-terminal prodomain for apelin-13 by PCSK3.

The observation of His-apelin-55 Δ 32 as the second most prevalent N-terminal domain fragment in HEK293A culture

medium is less straightforward to link to PCSK3 activity. One possibility follows from the identification of apelin-31 both in bovine colostrum and milk (Mesmin et al. 2011) and, herein, in 3T3-L1 culture medium (Fig. S15). Extracellular processing to apelin-31—by some as yet-unidentified route—would, in turn, result in a product, His-apelin-55 Δ 31, with a C-terminal Arg (Fig. 6) that could be processed by an Arg exoprotease to His-apelin-55 Δ 32 analogously to His-apelin-55 Δ 13 to His-apelin-55 Δ 15.

Consistent with the potential for a wide variety of extracellular processing events to occur, minor eluents were observed with masses corresponding to His-apelin-55 with both N-terminal (19–30 residue) and C-terminal (15–16 residue) truncations. The exact sources of the wide variety of peptide products observed are hard to determine, but are consistent with the extensive catalog of apelin peptides seen in colostrum and milk (Mesmin et al. 2011). Because none of the related apelin isoforms themselves (i.e., apelin-13, -15, -16, or -32) were detected in the medium, any additional discussion would be even more speculative in nature. However, it is important to note that the lack or low levels of observable C-terminal products is not unexpected given that bioactive C-terminal apelin peptides have been shown to undergo rapid degradation in plasma (Murza et al. 2014), and with rates of degradation by the metalloprotease neprilysin being greater for the apelin-13 and -17 isoforms relative to apelin-36 (McKinnie et al. 2016). Despite this shortcoming in delineating the exact mechanism(s) of processing taking place, these results collectively demonstrate the potential for PCSK-dependent extracellular apelin processing employing HEK293A cells as a tractable model system.

Interestingly, pretreatment of HEK293A cells with the PCSK inhibitor decanoyl-RVKR-CMK did not modify the level of His-apelin-55 Δ 15 resolved by HPLC, but resulted in the observation of a new major processing product consistent with His-apelin-55 Δ 1 (Fig. 6). Angiotensin-converting enzyme 2 (ACE2) (Vickers et al. 2002) and prolylcarboxypeptidase (PRCP) (Kehoe et al. 2016) have both been shown

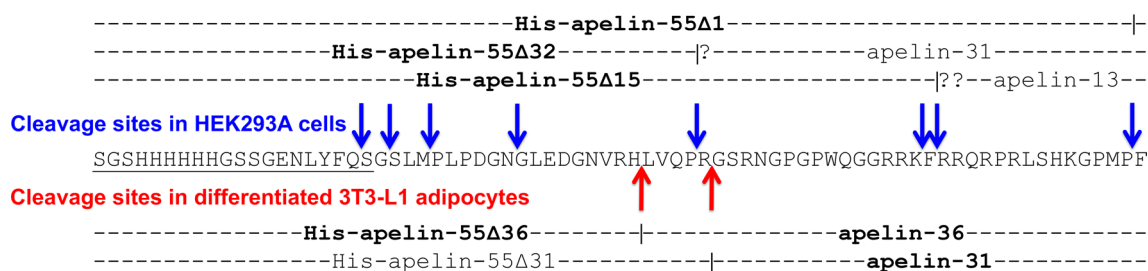


Fig. 6 Summary of His-apelin-55 processing based on cleavage products observed in HEK293A cell and 3T3-L1 adipocyte culture media. The primary observed N- and C-terminal processing products are delineated, with bold type lettering distinguishing products observed by RP-HPLC and ESI-MS under the assay conditions. Arrows indi-

cate all observed cleavage sites resolved upon incubation with each cell type. Question marks represent residues predicted to be removed as a result of further proteolytic cleavage. Underlined residues represent the N-terminal His₆ tag and TEV protease cleavage site

to remove the C-terminal Phe of apelin *in vitro*. This has, in turn, been linked with deactivation and regulation in the apelinergic system (Ceraudo et al. 2014; Wang et al. 2016). Both ACE2 and PRCP are located on the cell surface and are expressed in kidney cells (Tipnis et al. 2000; Shariat-Madar et al. 2002); thus, either (or both) may be responsible for the observed processing upon PCSK inhibition. Observation of this processed form only in the presence of inhibitor is notable for three reasons. First, observation of a major product other than His-apelin-55 Δ 15, resulting from a well-characterized mechanism, unequivocally demonstrates the involvement of other proteases and underlines the non-proportional changes observed between the level of intact His-apelin-55 and new processed products. Second, it shows a direct and efficient truncation of the apelin C-terminal residue in HEK293A cell culture, likely hampering the ability to detect intact apelin isoforms. Third, the similar level of His-apelin-55 Δ 15 observed with inhibitor treatment relative to without, despite the presence of an additional major processing product in the presence of inhibitor, further implies that the processes involved in regulation of His-apelin-55 Δ 15 levels are leading to steady-state behavior.

The 3T3-L1 adipocyte cell line has previously been employed for characterization of physiological roles of apelin such as adipogenesis, lipolysis, and glucose homeostasis (Than et al. 2012; Zhu et al. 2011). Thus, it is highly noteworthy that incubation of His-apelin-55 with 3T3-L1 adipocytes resulted in a clearly distinct processing pattern relative to with HEK293A cells, with the primary N-terminal cleavage product observed being His-apelin-55 Δ 36. Correspondingly, apelin-36 was directly detected. Apelin-31 was also detected, suggesting that multiple forms of processing take place and, potentially, tying to the observation of His-apelin-55 Δ 32 in HEK293A culture medium.

Despite endogenous PCSK3 expression in 3T3-L1 cells (Shin et al. 2013), these results imply that the processing of apelin in 3T3-L1 medium is likely primarily due to PCSKs other than PCSK3 or through PCSK-like endoproteases that are either not present or relatively inactive in HEK293A cells. Subsequently, this as yet-unidentified endoprotease may be present at relatively low levels or have higher sensitivity to decanoyl-RVKR-CMK, resulting in the tenfold decrease in minimum inhibitor concentration observed between cell lines.

As an alternative to extracellular processing, a mechanism could be envisioned where His-apelin-55 is internalized through receptor-mediated endocytosis or another endocytic pathway, processed intracellularly, and then secreted. Such mechanisms would be highly energy intensive and are difficult to reconcile with the degree of apelin processing observed in both culture media. Correspondingly, we are unaware of such a mechanism having been previously observed. Furthermore, examples of exactly the opposite

behavior have been documented, where proproteins are secreted and specifically rely on processing by cell surface PCSK3 (Koo et al. 2006; Xiao et al. 2008). Receptor-mediated trafficking and subsequent intracellular processing is also unlikely to occur, given, as noted above, the negative correlation of apelin-AR dissociation to isoform length and the fact that apelin-36 exhibits a sustained receptor interaction leading to receptor degradation (Lee et al. 2010). Observation of an anionic surface on the AR extracellular domain (Ma et al. 2017) is correlated with this potential for favourable ligand-receptor binding (Shin et al. 2018). Apelin-55, thus, seems likely to follow the precedent of apelin-36 with favourable binding to the receptor that would prevent processing through steric hindrance, occlusion of the proteolytic site, and/or limiting of the quantity of free apelin-55. Furthermore, while 3T3-L1 adipocytes express AR (Attane et al. 2011), HEK293A cells do not (Bai et al. 2014a, b). An AR-mediated mechanism of internalization would thus be possible for 3T3-L1 adipocytes, but not HEK293A cells.

In conclusion, extracellular apelin processing is both possible and highly probable. Through extracellular processing, a mechanism may be envisioned where longer apelin isoforms (e.g., apelin-55 or -36) in circulation are processed to specific isoform(s) at an appropriate location to exert the isoform-specific physiological responses observed previously (Adam et al. 2016; Galon-Tilleman et al. 2017). This is also consistent with preferential tissue-dependent apelin isoform enrichment (Kawamata et al. 2001; De Mota et al. 2004). Given the known differences in potency, efficacy, and in downstream effects, controlling apelin processing through the activity of specific cell surface enzymes would serve to provide an additional level of regulation in directing downstream effects. Such processing-mediated regulation may not be limited to apelin, with the recent identification of apela (ELABELA (Chng et al. 2013)/Toddler (Pauli et al. 2014)) as a second endogenous peptidic ligand for the AR. Like apelin, apela can be processed into multiple N-terminally truncated forms with isoform-dependent functional properties (Shin et al. 2018), implying the potential for similar regulation. In combination with the previously demonstrated potential of intracellular apelin processing, extracellular processing of apelinergic system ligands is indicative of the involvement of diverse mechanisms and proteases in regulation of the apelinergic system.

Acknowledgements Thanks to Xiao Feng (Dalhousie Mass Spectrometry Laboratory) and Bruce Stewart for their expert technical support. This work was supported by Canadian Institutes of Health Research (CIHR) Operating Grants (MOP-111138 to JKR and MOP-82795 to YA) and the IWK Research Foundation (Category B to YA). Key equipment was provided through the Natural Sciences and Engineering Research Council of Canada (NSERC) and Dalhousie Medical Research Foundation. KS held an NSERC Alexander Graham Bell Canadian Graduate Scholarship and a Killam Predoctoral Scholarship; JKR was supported by a CIHR New Investigator Award.

Compliance with ethical standards

Conflict of interest The authors report no relationships or interests that would lead to a bias or undue influence.

Research involving human participants and/or animals None.

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