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Urotensin-II converting enzyme activity of furin and trypsin in human cells *in vitro*

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Running Title: Human urotensin converting enzymes

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Abbreviations: hU-II, human urotensin-II; CTF-ProhU-II, 25 amino acid residue carboxyl terminal fragment of urotensin-II prohormone; UCE, urotensin converting enzyme.

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Abstract

Human urotensin-II (hU-II) is processed from its' prohormone (ProhU-II) at putative cleavage sites for furin, and serine proteases such as trypsin. While proteolysis is required for biological activity, the endogenous "urotensin converting enzyme" (UCE) has not been investigated. The aim of this study was to investigate UCE activity in cultured human cells and in blood, comparing activity to that of furin and trypsin. In a cell-free system, hU-II was detected by high performance liquid chromatography-mass spectrometry after co-incubating 10 μM CTF-ProhU-II with recombinant furin (2U/ml, 3h, 37°C) at pH 7.0 and pH 8.5, but not at pH 5.0, or when the incubating medium was depleted of Ca^{2+} ions and supplemented with 2 mM EDTA at pH 7.0. hU-II was readily detected in the superperfusate of permeabilised epicardial mesothelial cells incubated with CTF-ProhU-II (3h, 37°C), but was only weakly detected in the superperfusate of intact cells. Conversion of CTF-ProhU-II to hU-II was attenuated in permeabilised cells using conditions found to inhibit furin activity. In a cell-free system, trypsin (0.05 mg/ml) cleaved CTF-ProhU-II to hU-II and this was inhibited with 35 μM aprotinin. hU-II was detected in blood samples incubated with CTF-ProhU-II (3h, 37°C) and this was also inhibited with aprotinin**.** The findings revealed an intracellular UCE in human epicardial mesothelial cells with furin-like activity. Aprotinin-sensitive UCE acitivity was detected in blood, suggesting that an endogenous serine protease such as trypsin may also contribute to proteolysis of hU-II prohormone, if the prohormone is secreted into the circulation.

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Introduction

Human urotensin-II (hU-II) is an endogenous peptide ligand that interacts with the recently cloned human G-protein coupled receptor, UT-II. The UT-II receptor system is ubiquitously expressed in mammalian tissues, including heart, lungs, blood vessels, kidney and brain. The vascular effects of receptor stimulation include contraction of isolated endothelium-denuded arteries (Maguire et al., 2000; Russell et al., 2001) and veins (Maguire et al., 2000), and endothelium- and nitric oxide-dependent relaxation of small human pulmonary- and rat renal arteries (MacLean et al., 2000; Zhang et al., 2003). Infusion of hU-II into the renal artery of anaesthetized rats caused an increase in renal blood flow (Zhang et al., 2003), consistent with relaxation of the isolated renal artery preparation. In humans, intra-brachial artery infusion of hU-II either reduced (Böhm & Pernow, 2002), or had no effect (Wilkinson et al., 2002) on forearm blood flow. Infusion of hU-II into anaesthetized monkeys caused cardiodepression (Ames et al., 1999), possibly secondary to massive vasoconstriction as hU-II caused positive inotropy in isolated human right atrial and ventricular strips (Russell et al., 2001).

Two alternative splicing variants of human prohormone (prepro-hU-II), consisting of 124 and 139 amino acid residues, have been cloned and these were found to differ only in their amino terminus (Coulouarn et al., 1998; Ames et al., 1999). The conserved, carboxyl terminus of the prohormone is cleaved to produce a mature, 11 amino acid residue peptide. The potency of this mature peptide was markedly higher than a larger C-terminal fragment of the prohormone (CTF-ProhU-II; Russell et al., 2003), suggesting that proteolytic cleavage is required for biological activity. Despite this requirement, the pathways leading to the production of hU-II are poorly understood.

For example, it is not known which cells contain enzymes that may contribute to proteolytic cleavage of the prohormone, or whether this processing occurs at the cell surface or intracellularly.

Direct evidence for the expression of a "urotensin converting enzyme" was demonstrated in porcine renal tissue using a mass spectrometry-assisted enzyme screening system (Schlüter et al., 2003), although the identity of the convertase involved was not investigated. Multiple high molecular weight urotensin-II-like peptides were extracted from the superperfusate of cultured SW-13 adrenocortical carcinoma cells, indicating possible proteolytic processing of the prohormone by these cells (Takahashi et al., 2001). More recently, molecular biology techniques revealed localization of prepro-U-II mRNA in mammalian cells, including human vascular smooth muscle cells (Douglas et al., 2002), endothelial cells (Douglas et al., 2002; Totsune et al., 2003), endocardial endothelial cells (Douglas et al., 2002) and motoneurones (Coulouarn et al., 1998), and rat cardiac fibroblasts (Tzanidis et al., 2000). It remains to be determined whether the prohormone is processed locally within these cells, or is secreted and processed at a remote location.

Putative post-translational cleavage sites have been identified within the urotensin-II prohormone, including multiple mono- and polybasic amino acid residues (Conlon et al., 1990; Douglas et al., 2000). It is interesting to note that the R^{12}-K^{13}-K^{14}-Y^{15}-P^{16}-K^{17} amino acid sequence of the urotensin-II prohormone also conforms to stringent rules that govern the efficiency of convertase activity by furin, an enzyme that is expressed primarily in the *trans*-Golgi network of most cell types, and with a role in proteolytic cleavage of a large number of prohormones (Nakayama, 1997).

The circulation may present an alternate site for urotensin converting enzyme activity. Urotensin-II-like immunreactivity has been detected in blood from healthy individuals and patients with renal and cardiovascular disease (Totsune et al., 2001; Richards et al., 2002; Russell et al., 2003; Totsune et al., 2003). However, antibodies used in the detection of mature hU-II cross-reacted with CTF-ProhU-II (Russell et al., 2003), raising the possibility that full-length, or partially processed prohormone might contribute to immunoreactivity detected in blood. Monobasic and dibasic residues form common recognition sequences for broad range peptide and protein convertases. In this study, we focus on the enzymatic activity of trypsin, an enzyme present in serum of normal healthy individuals (Le Moine et al., 1994) and whose substrate specificity for X^1-K^2 and X^1-R^2 bonds would be predicted to cleave between the E^{11}-R^{12} residues of prohU-II. The aim of this study was to investigate UCE activity in cultured human cells and in blood, comparing activity to that of furin and trypsin.

Methods

Collection of tissues

Human epicardial mesothelial cells and fibroblasts were cultured from right atrial appendages obtained from patients undergoing coronary artery bypass graft surgery for coronary artery disease. After surgical removal, atrial trabeculae were placed immediately in ice-cold, pre-oxygenated (95% O_2/5% CO_2) modified Krebs' solution (125 mM Na^+, 5 mM K^+, 2.25 mM Ca^{2+}, 0.5 mM Mg^{2+}, 98.5 mM Cl^-, 0.5 mM SO_4^{2-}, 32 mM HCO_3^-, 1 mM HPO_4^{2-}, 0.04 mM EDTA). Human endothelial cells were cultured from umbilical vein. Umbilical cords were collected in phosphate buffered solution (1.54 mM KH_2PO_4, 155 mM NaCl, 2.7 mM $Na_2HPO_4.2H_2O$; pH 7.2). Human right atria and umbilical cords were collected from patients giving informed consent, and with approval from the ethics committees of The Prince Charles Hospital (EC2134), The Royal Women's Hospital (RWH 01/22) and from The University of Queensland (2001002160; 2002000008). Forearm venous blood samples were obtained from healthy volunteers.

Cell culture

Right atrial appendages were immersed in Hanks buffered saline solution containing 0.25% trypsin and 1 mM EDTA (30 min, RT), and mesothelial cells were dislodged from the epicardium using a cell scraper. The cell suspension was spun (230 x g, 6 min, 4°C), the pellet resuspended in Dulbecco's Modified Eagle Medium containing 20% fetal bovine serum, penicillin (100 U/ml) and streptomycin (0.1 mg/ml) (20% DMEM solution), and plated into 6 well plates. Cryostat cut sections (20 μm) of the atrium were collected on microscope slides and stained with cresyl violet to verify

removal of epicardial mesothelial cells. The atrial appendage was scraped vigorously with a scalpel blade to remove any residual mesothelium, and blocks of epicardium (≈0.5 mm^3) were transferred to 6 well plates and incubated in 10% DMEM solution. After 2-3 weeks, blocks of tissue were removed from the plates and fibroblasts, which had proliferated from the blocks, were collected using Trypsin-Versene Mixture (Bio-Whittaker, Rockland, USA). The fibroblast cell suspension was spun (230 x g, 6 min, 4°C), the pellet resuspended in 10% DMEM solution and plated into 6 well plates.

Umbilical veins from human umbilical cords were cannulated with a blunt end needle and flushed with DMEM to remove blood. The non-cannulated end of the cord was clamped and DMEM containing 1mg/ml collagenase IV was administered into the vein and incubated for 30 min at RT. The cell suspension was collected, spun (230 x g, 6 min, 4°C), resuspended in 20% DMEM solution and plated into 6 well gelatin coated (2.5 mg/ml) plates.

Primary cultures of all cell types were used to minimize phenotypic changes, and cells were grown to confluence in a 5% CO_2 incubator. Culture medium was changed every three days. Cells were also grown on gelatin-coated glass coverslips for immunocytochemistry. Mesothelial cells stained positively with a mouse monoclonal antibody to cytokeratin 18 (clone B23.1; 1:100; Abcam, Cambridge, UK), fibroblasts with a mouse monoclonal antibody to SM1214 (clone D7-FIB; 1:500; DPC Biermann, Bad Nauheim, Germany), and endothelial cells with a mouse monoclonal antibody to von Willebrand factor (clone F8/86; 1:100; Dako, Glostrup, Denmark). Fibroblasts stained negatively with a mouse monoclonal antibody to smooth muscle α-actin (clone 1A4; 1:1000; Immunon, Pittsburgh, USA). Immunoreactivity was detected using an

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anti-mouse IgG Texas Red conjugate (20 μg/ml; Molecular Probes, Leiden, The Netherlands), and viewed using an Olympus CK2 inverted microscope.

Biochemical studies

CTF-ProhU-II (10 μM, Auspep Pty Ltd, Parkville, Australia) was incubated for 3h at 37°C in incubation medium containing Tris HCl (100 mM; pH 5.0, 7.0 or 8.5) and $CaCl_2$ (1 mM), with or without furin (2U/ml; New England BioLabs, Beverly, USA), trypsin (0.05 mg/ml; Type XI; Sigma, St. Louis, USA), or Triton X-100 (0.1%). Samples containing furin were incubated with or without medium that was depleted of Ca^{2+} ions and supplemented with 2 mM EDTA, while samples containing trypsin were incubated in the absence or presence of 34.5 μM aprotinin.

In a separate series of experiments, confluent cultured human epicardial mesothelial cells (n=3), vascular endothelial cells (n=4) and cardiac fibroblasts (n=3) (2 wells/treatment) were incubated with CTF-prohU-II (10 μM) in incubation medium containing Tris HCl (100 mM; pH 5.0, 7.0 or 8.5), with or without $CaCl_2$ (1 mM) and EDTA (2 mM). Cells were co-incubated in the absence (non-permeabilised cells) or presence of 0.1% Triton X-100 (permeabilised cells) for 3h at 37°C.

Blood collected from healthy individuals (n=3) was immediately incubated with CTF-ProhU-II (25 μM) and Tris HCl (100 mM; pH 7.0) in the absence or presence of 34.5 μM aprotinin and 0.1 % Triton X-100. A sample of freshly collected blood was also spun (1,600 x g, 15 min, 4°C), plasma was collected and incubated with CTF-ProhU-II (25 μM) and Tris HCl (100 mM; pH 7) in the absence or presence of 34.5 μM aprotinin. Control experiments were carried out in the absence of CTF-ProhU-II.

All samples were loaded onto Sep-Pak C18 cartridges (Waters Co., Taunton, USA) which were pre-equilibrated with 0.1% trifluoroacetic acid (10 ml; solvent A), 90% acetonitrile in 0.1% trifluoroacetic acid (10 ml; solvent B), and 10 ml of solvent A. Samples were washed with 3 ml solvent A and eluted with a 2:3 ratio mixture of solvents A and B. The samples were evaporated to dryness in a Savant Speed Vac SC210a and then reconstituted with solvent A (0.1 ml) for HPLC-MS analysis.

Liquid Chromatography - Mass Spectrometry (LCMS)

Gradient high performance liquid chromatography analyses were performed on an Agilent SB-C18 (2.1 x 50 mm) column at a flow rate of 0.3 ml/min. The gradient used was solvent A (100%, 2 min), then ramped from 100% solvent A to 80% solvent B over 8 min, held at 80% solvent B for 2 min then returned to 100% solvent A in 6 s. The column was finally washed with 100% solvent A for a further 6 min. Solvents for the binary gradient were delivered with Shimadzu LC10 AT VP liquid chromatography (LC) pumps that were controlled with a SCL10A VP Shimadzu system controller. Solvents were degassed with a DGU12A Shimadzu degasser, and samples were introduced to the column using an Agilent 1100 series auto injector. Detection was achieved using a PE Sciex API3000 LC-MS-MS system. Samples were ionized by positive ion electrospray atmospheric pressure ionization. The ion spray, orifice (O_r) and ring voltages were 5200, 35 and 225 V, respectively. The quadrupole zero (Q_o) voltage was -10V, and the declustering potential was 45V (O_r-Q_o).

An authentic sample of synthetic hU-II (Auspep Pty Ltd, Parkville, Australia) was analysed by LCMS and the retention time of hU-II was found to be 6.7 min (Figure 2). hU-II was detected as a doubly charged ion (m/z 694.4 $[M+2H]^{2+}$). An extracted

ion chromatogram (m/z 693-696) was generated to define the hU-II peak. The hU-II peak was integrated to allow semi-quantitative determination of hU-II (Figure 3), and the area data was expressed as total ions counted. The sensitivity of detection for hU-II and CTF-ProhU-II was 1 pmole.

Results

Investigation of furin and trypsin in the proteolytic processing of CTF-ProhU-II

The proteolytic processing of prepro-hU-II was investigated by determining the formation of mature hU-II peptide from a 25 amino acid residue, carboxy-terminal sequence that is conserved in the full-length prohormone splice variants (CTF-ProhU-II; Figure 1). CTF-ProhU-II and hU-II were separated using high performance liquid chromatography, with retention times of 5.9 and 6.7 min, respectively (Figure 2A). The mass to charge ratios (m/z) for CTF-ProhU-II and hU-II, determined using mass spectrometry were 634.2 ($[M+5H]^{5+}$) and 694.4 ($[M+2H]^{2+}$), respectively (Figure 2B, 2C). Semi-quantitative analysis of hU-II levels in cell-free and biological samples was determined after generation of an extracted ion chromatogram (m/z 693-696, Figure 3).

In a cell-free system, furin and trypsin cleaved CTF-ProhU-II to produce mature hU-II peptide. The processing of CTF-ProhU-II by furin was pH-dependent, with efficient converting enzyme activity evident at pH 7.0 (Figures 4A, 4B, 4M) and pH 8.5 (Figure 4C, 4D), but not at pH 5.0 (Figures 4E, 4F, 4M). Proteolytic conversion of CTF-ProhU-II was markedly reduced at pH 7.0 when the incubation buffer was depleted of Ca^{2+} ions and supplemented with 2 mM EDTA (Figures 4G, 4H, 4M). CTF-ProhU-II was also cleaved by trypsin (Figure 4I, 4J, 4M), and this was sensitive to the trypsin inhibitor, aprotinin (Figures 4K, 4L, 4M).

UCE resembles furin-like activity in vascular and cardiac cells

Expression of a cell surface "urotensin-II converting enzyme" was investigated by measuring the conversion of CTF-ProhU-II to hU-II by cultured, non-permeabilised human epicardial mesothelial cells, cardiac fibroblasts and umbilical vein endothelial

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cells. Only low levels of mature hU-II were detected in the superperfusate of mesothelial cells (Figure 5A, 5B, 5M), fibroblasts (not shown) and endothelial cells (not shown), suggesting that an ectoenzyme is not a major contributor to prohormone processing by these cells. However hU-II was readily detected in cell superperfusates when cells were incubated with CTF-ProhU-II and permeabilised with 0.1% Triton X-100 (Figures 5C, 5D, 5M). hU-II was not detected when permeabilised cells were incubated without CTF-ProhU-II (Figures 5E, 5F, 5M), and was only weakly detected in a cell-free system in which prohU-II was incubated with 0.1% Triton X-100 (Figures 5G, 5H, 5M), suggesting that mature peptide was not released from intracellular compartments following cell permeabilisation, and that the presence of hU-II could not be attributed to spurious cleavage of CTF-ProhU-II by the cell permeabilisation procedure.

Consistent with the characteristics of the activity of recombinant furin, the intracellular "urotensin converting enzyme" was inhibited, although not abolished under low pH conditions (pH 5.0; Figure 5I, 5J, 5M), and by the substitution of incubation medium that was depleted of Ca^{2+} ions and supplemented with EDTA (Figure 5K, 5L, 5M). The partial insensitivity of enzymatic activity to these conditions may also indicate redundancy with respect to intracellular urotensin converting enzymes.

Evidence for trypsin-like UCE activity in the circulation

hU-II was readily detected in samples of whole blood that were incubated with CTF-ProhU-II (Figure 6A, 6B, 6E). hU-II was not detected in blood incubated without ProhU-II (not shown), consistent with the low sensitivity of detection of mass spectrometry for endogenous hU-II in blood from individuals with non-failing hearts.

Processing of CTF-ProhU-II by whole blood was sensitive to aprotinin, suggesting the presence of a trypsin-like serine protease in the circulation that is capable of processing the prohormone (Figure 6C, 6D, 6E). Similar findings were obtained when CTF-ProhU-II was incubated with plasma (not shown), indicating that the circulating "urotensin converting enzyme" was not associated with erythrocytes.

HU-II-like immunoreactive staining in human epicardial mesothelial cells

Cultured human epicardial mesothelial cells were positively identified using the mesothelial cell marker, anti-cytokeratin 18 (Figure 7A). Positive staining for anti-hU-II peptide was observed in the cytoplasm of the cultured cells, in the perinuclear region (Figure 7B). Negligible levels of staining were detected over the nucleus (Figure 7B), and in control cells incubated without antibody (Figure 7C).

Discussion

Proteolytic cleavage of prohU-II is required for biological activity (Russell et al., 2003), and so the aim of this study was to investigate "urotensin converting enzyme" activity in cultured human epicardial mesothelial cells, vascular endothelial cells and cardiac fibroblasts, and in whole blood and plasma samples, comparing activity to that of furin and trypsin. Proteolytic enzyme activity was investigated by examining the conversion of a 25 amino acid C-terminal fragment of prohU-II (CTF-ProhU-II) to mature peptide.

Multiple putative post-translational cleavage sites have been identified within preprohU-II (Conlon et al., 1990; Douglas et al., 2000), including a pair of basic amino acid residues, R^{12}-K^{13} positioned at the convertase site. While dibasic residues are a common recognition sequence for broad range peptide and protein convertases, it is interesting to note that the E^{11}-R^{12}-K^{13}-K^{14}-Y^{15}-P^{16}-K^{17} amino acid sequence of the prohormone also conforms to stringent rules that govern efficient prohormone convertase activity of furin: an Arg residue at P_1 (R^{12}), basic residues in at least two positions corresponding to P_2, P_4 and P_6 (K^{13}, K^{17}), and a non-hydrophobic aliphatic side chain at P_1' (E^{11}) (Nakayama, 1997). Furin is a ubiquitously expressed enzyme in mammalian cells, primarily localized to the *trans*-Golgi network, and with a role in endoproteolysis of a number of precursor hormones (Nakayama, 1997). We predicted that prohU-II would be a substrate for furin, a hypothesis supported by our observation that in a cell-free system, recombinant furin cleaved CTF-ProhU-II to mature hU-II. Furin has a requirement for Ca^{2+} ions (optimal concentration is 1.0 mM) and is inactive in Ca^{2+}-free medium, and is inhibited by 2.0 mM EDTA (Molloy et al., 1992). Optimal furin activity was observed in the pH range 7.0 to 8.5, with little or no activity at pH 5.0

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(Molloy et al., 1992). In the cell-free system in this study, recombinant furin cleaved CTF-ProhU-II at pH 7.0 and 8.5, but was markedly reduced at pH 5.0. At pH 7.0, conversion of CTF-ProhU-II was also markedly reduced using medium supplemented with 2.0 mM EDTA and depleted of Ca^{2+} ions, consistent with the previously described characteristics of furin activity.

Proteolytic conversion of CTF-ProhU-II to hU-II was attenuated, although not abolished in permeabilised cells under conditions designed to inhibit furin activity. This finding suggested that although furin may cleave the urotensin-II prohormone in cells, other intracellular enzymes capable of processing the prohormone might contribute to the observed UCE activity. A limitation of the study is that permeabilisation of cells with Triton X-100 caused disruption to cell surface and all intracellular membranes, resulting in exposure of CTF-ProhU-II to intracellular enzymes that may not otherwise come in contact with the endogenous prohormone. However, furin has a critical role in the processing of many prohormones during their transit to the cell surface (Nakayama, 1997), and it is likely that the urotensin-II prohormone would also traverse the *trans*-Golgi network. Consistent with this hypothesis, positive immunoreactive staining for hU-II was observed in the perinuclear region of epicardial mesothelial cells.

Urotensin-II-like immunoreactivity has been detected by radioimmunoassay or ELISA in human plasma (Heller et al., 2001; Totsune et al., 2001; Ng et al., 2002; Richards et al., 2002; Russell et al., 2003; Totsune et al., 2003), however it is not known to what extent, if any the prohormone or fragments of the prohormone contribute to this immunoreactivity. The ability of hU-II antibodies to cross-react with CTF-ProhU-II (Russell et al., 2003) raised the possibility that the prohormone or fragments of the

prohormone might be secreted into the circulation, as has been shown for other peptide systems, for example endothelin (ET-1, big ET-1; Neri Serneri et al., 2000), adrenomedullin (AM, PAMP; Samson, 1998) and brain natriuretic peptide (BNP-32, high molecular weight BNP, NT-proBNP; Hunt et al., 1997). Although elevated plasma levels of hU-II-like immunoreactivity were detected by radioimmunoassay in patients with congestive heart failure compared to patients with non-failing hearts, mature hU-II was not detected using mass spectrometry (Russell et al., 2003), raising the possibility that the antibody recognised a circulating prohormone, a prohormone fragment, or a distinct peptide with sequence similarity to hU-II. Interestingly, cDNA encoding a "urotensin-II-related peptide" was recently cloned from rat, mouse and human, where the putative convertase site also contained dibasic amino acid residues (Sugo et al., 2003). Although overall amino acid sequence homology was only 18.8% between preprohU-II and the "urotensin-II-related peptide", high sequence homology was observed in the C-terminal region that is crucial for both biological activity and immunochemical studies.

If prohU-II is secreted into the circulation, proteolytic conversion is unlikely to occur at the intimal surface of blood vessels because only very modest levels of hU-II were detected in the superperfusate of cultured, non-permeabilised endothelial cells that were incubated with CTF-ProhU-II (this study). We therefore investigated the possible cleavage of CTF-ProhU-II in blood, focusing on the enzymatic activity of trypsin, whose substrate specificity for X^1-K^2 and X^1-R^2 bonds would be predicted to cleave between the E^{11}-R^{12} residues of prohU-II. Trypsinogen, an inactive zymogen is secreted from the pancreas into the gut where it is converted to trypsin by enterokinase. Trypsin is present within the serum of normal healthy individuals, where the activity is

about 80 Units/litre (Le Moine et al., 1994). The ability of trypsin to produce mature peptide from CTF-ProhU-II was demonstrated in the present study using a cell-free system, where proteolytic processing was sensitive to the trypsin inhibitor, aprotinin. In blood, CTF-ProhU-II was converted to hU-II and this was also sensitive to inhibition by aprotinin, raising the possibility that a serine protease such as trypsin may be an endogenous, circulating "urotensin-II converting enzyme". Processing of CTF-ProhU-II was independent of erythrocytes because similar findings were also observed for samples of plasma.

In conclusion, this study investigated the characteristics of the "urotensin converting enzyme" in human epicardial mesothelial cells, vascular endothelial cells and cardiac fibroblasts and in human whole blood and plasma samples by examining conversion of a C-terminal fragment of prohU-II to hU-II using HPLC / mass spectrometry. The findings revealed that cells contained an intracellular enzyme with furin-like characteristics capable of processing CTF-ProhU-II to hU-II. Only very modest converting enzyme activity was detected on the cell surface. hU-II was detected in blood samples incubated with CTF-ProhU-II, and this was inhibited with aprotinin, suggesting that an endogenous serine protease such as trypsin may also contribute to proteolysis of prohU-II, if the prohormone is secreted into the circulation.

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Footnotes

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Figure 1 Diagramatic representation of the proteolytic conversion of C-terminal fragment of pro-human urotensin-II (CTF-ProhU-II) to biologically active human urotensin-II (hU-II) by urotensin converting enzyme (UCE).

Figure 2 High performance liquid chromatography (HPLC) showing total ions counted per second (cps) against retention time, and mass spectral data showing relative ion intensity against the mass to charge ratio (m/z) for CTF-prohU-II and hU-II. Retention times for CTF-prohU-II (arrow head) and hU-II (arrow) were 5.9 and 6.7 min, respectively using HPLC (A). The mass to charge ratios were CTF-prohU-II: 634.2 ($[M+5H]^{5+}$) (B), and hU-II: 694.4 ($[M+2H]^{2+}$) (C).

Figure 3 Illustration of the procedure used for semi-quantitative determination of hU-II present in the biological samples. hU-II eluted from the column with a retention time of 6.7 min (arrow, A), consistent with authentic hU-II (see Figure 2). An extracted ion chromatogram (m/z 693-696) was generated to define the hU-II peak (B), and this was integrated to determine the amount of hU-II in the sample. The presence of hU-II, detected as a doubly charged ion (monoisotopic ^{12}C peak, m/z, 694.4 $[M+2H]^{2+}$) was confirmed by mass spectrometry (C). The x-axis was expanded to reveal peaks for $m+^{13}C/z$, 694.9 and $m+2.^{13}C/z$, 695.4.

Figure 4 Relative amount of hU-II formed from CTF-ProhU-II in a cell free-system, determined by mass spectrometry. hU-II was detected after incubation of CTF-ProhU-II with 2U/ml furin at pH 7.0 and pH 8.5 (not shown), but not at pH 5.0, or at pH 7.0 in Ca^{2+}-depleted/ EDTA supplemented medium. hU-II was readily detected in cell-

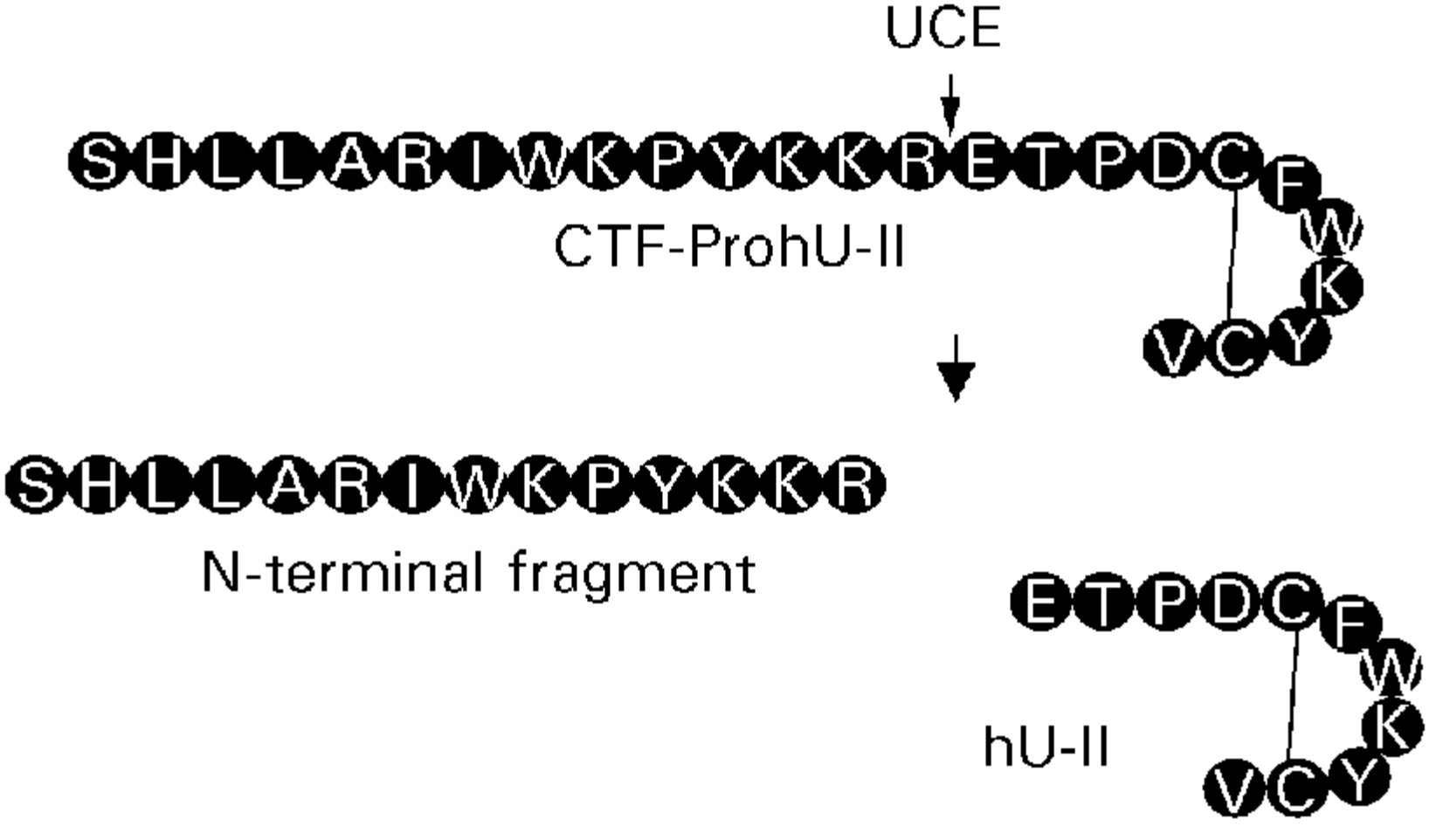
free medium containing CTF-ProhU-II which was incubated with 0.05 mg/ml trypsin, but was undetected following co-incubation with trypsin and 34.5 μM aprotinin. Data is expressed as total number of ions counted over the period of the hU-II peak. Values are mean±s.e. mean, n=3; **, P<0.01.

Figure 5 Relative amount of hU-II formed from CTF-ProhU-II in mesothelial cell cultures and in a cell free-system, determined by mass spectrometry. hU-II was at the limit of detection in non-permeabilised mesothelial cell superperfusates at pH 7.0, but was readily detected in cells permeabilised with 0.1% Triton X-100. hU-II was not detected in permeabilised cells incubated without CTF-ProhU-II, and was detected, albeit at low levels when CTF-ProhU-II was incubated with Triton X-100 in a cell-free system. Formation of hU-II was attenuated, but not abolished in permeabilised mesothelial cells incubated at pH 5.0, and at pH 7.0 in Ca^{2+}-depleted/ EDTA supplemented medium. Data is expressed as total number of ions counted over the period of the hU-II peak. Values are mean±s.e. mean, n=3; **, P<0.01; ***, P<0.001.

Figure 6 Relative amount of hU-II formed from CTF-ProhU-II in venous blood that was obtained from healthy individuals, determined by mass spectrometry. hU-II was detected in whole blood incubated with CTF-ProhU-II. Formation of hU-II was attenuated, but not abolished in blood incubated with CTF-ProhU-II in the presence of 34.5 μM aprotinin. Data is expressed as total number of ions counted over the period of the hU-II peak. Values are mean±s.e. mean, n=3; *, P<0.05.

Figure 7 Immunocytochemical labelling of cultured human epicardial mesothelial cells. Epifluorescence staining for cytokeratin 18 (A), and positive hU-II-like immunoreactive staining in the perinuclear region of cultured epicardial mesothelial cells (B). Staining in the absence of primary antibody was negligible (C). Scale bars= 200 μm.

Figure 1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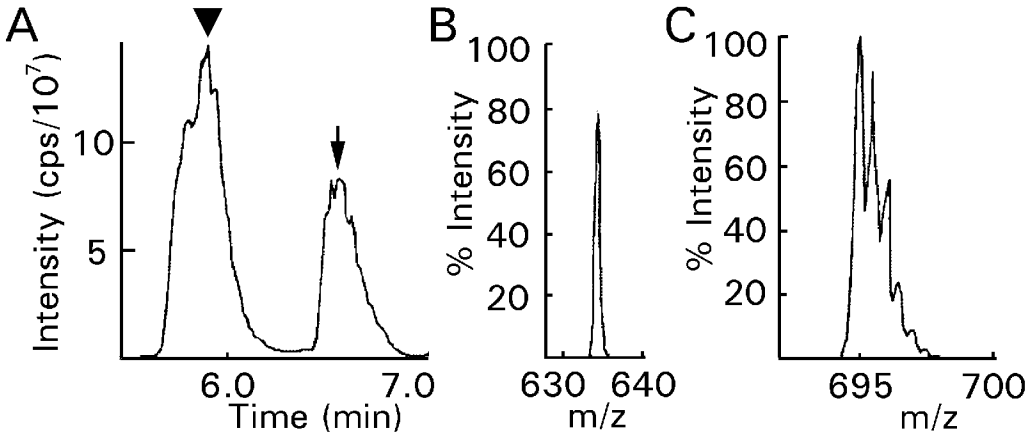
N-terminal fragment
ETPDCFWKVCY
hU-II

Figure 2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5
6.0
7.0
Time (min)
B
% Intensity
100
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60
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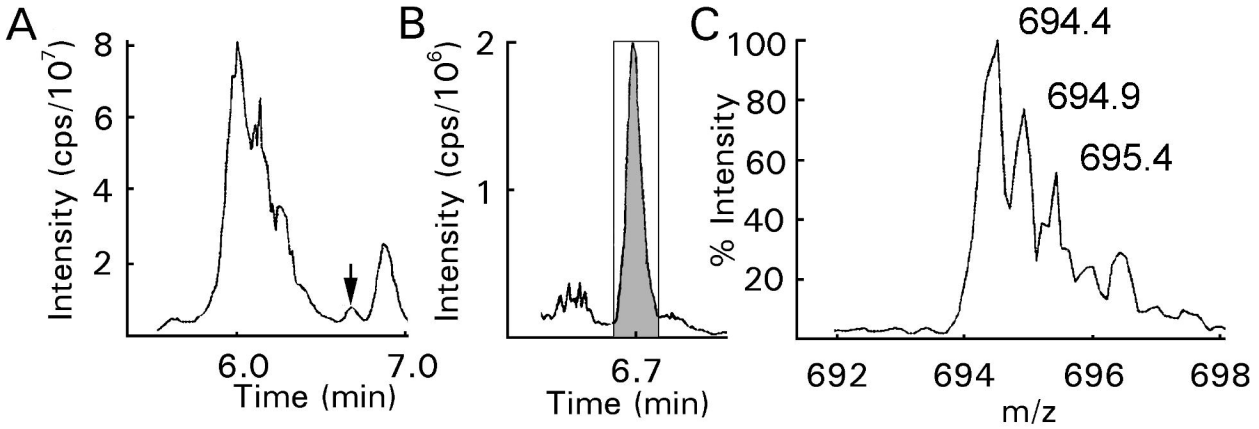
20
630
640
m/z
C
% Intensity
100
80
60
40
20
695
700
m/z

Figure 3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6
4
2
6.0
7.0
Time (min)
B
Intensity (cps/10^6)
2
1
6.7
Time (min)
C
% Intensity
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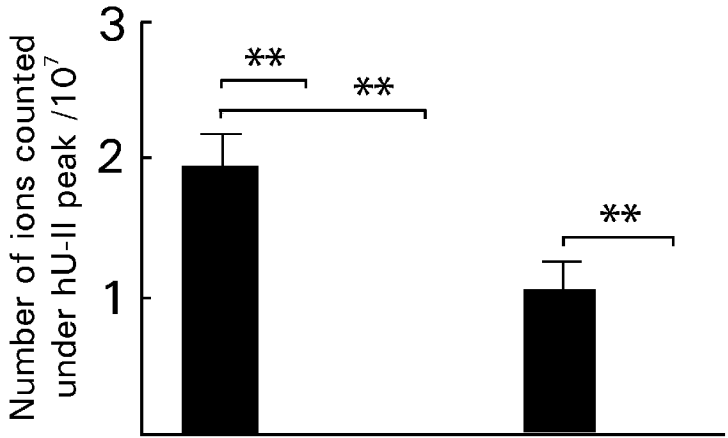
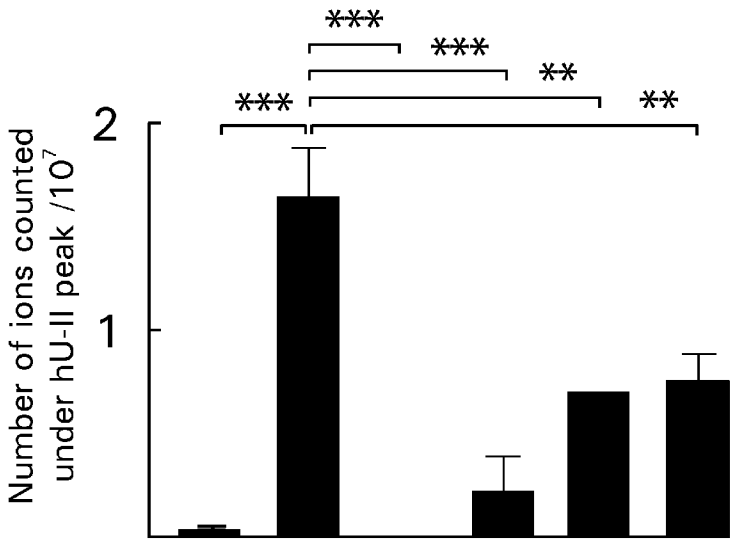
60
40
20
694.4
694.9
695.4
692
694
696
698
m/z

Figure 4



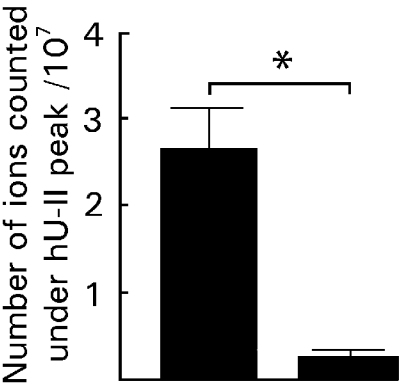
pH	7.0	5.0	7.0	7.0	7.0
25 μM CTF-ProhU-II	+	+	+	+	+
0.1% Triton X-100	+	+	+	−	−
1 mM Ca^{2+}	+	+	−	+	+
2 mM EDTA	−	−	+	−	−
2 U/ml Furin	+	+	+	−	−
0.05 mg/ml Trypsin	−	−	−	+	+
34.5 μM Aprotinin	−	−	−	−	+

Figure 5



pH	7.0	7.0	7.0	7.0	5.0	7.0
25 µM CTF-ProhU-II	+	+	−	+	+	+
0.1% Triton X-100	−	+	+	+	+	+
1 mM Ca^{2+}	+	+	+	+	+	−
2 mM EDTA	−	−	−	−	−	+
Mesothelial cells	+	+	+	−	+	+

Figure 6



pH	7.0	7.0
25 μM CTF-ProhU-II	+	+
0.1% Triton X-100	−	−
1 mM Ca^{2+}	+	+
2 mM EDTA	−	−
34.5 μM Aprotinin	−	+
Whole Blood	+	+

Figure 7