Urotensin-II-Converting Enzyme Activity of Furin and Trypsin in Human Cells in Vitro

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Received January 11, 2004; accepted March 8, 2004

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. Although 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 with that of furin and trypsin. In a cell-free system, hU-II was detected by high-performance liquid chromatography-mass spectrometry after coincubating 10 μM carboxyl terminal fragment (CTF)-ProhU-II with recombinant furin (2 U/ml, 3 h, 37°C) at pH 7.0 and pH 8.5, but not at pH 5.0, or when the incubating medium was depleted of Ca2+ ions and supplemented with 2 mM EDTA at pH 7.0. hU-II was readily detected in the superfusate of permeabilized epicardial mesothelial cells incubated with CTF-ProhU-II (3 h, 37°C), but it was only weakly detected in the superfusate of intact cells. Conversion of CTF-ProhU-II to hU-II was attenuated in permeabilized 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 (3 h, 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 activity 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.

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 anesthetized monkeys caused cardiodepression (Ames et al., 1999), possibly secondary to massive vasoconstriction because 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 (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

ABBREVIATIONS: hU-II, human urotensin-II; pro-hU-II, urotensin-II prohormone; CTF-ProhU-II, 25-amino acid residue carboxyl terminal fragment of urotensin-II prohormone; DMEM, Dulbecco’s modified Eagle’s medium; HPLC, high-performance liquid chromatography; UCE, urotensin-converting enzyme; BNP, brain natriuretic peptide.
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 superperfusionate 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 motoneurons (Couloarn et al., 1998), and rat cardiac fibroblasts (Tzaniidis 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 and Ohlstein, 2000). It is interesting to note that the R112-K113-K114-Y115-P116-K117 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 immunoreactivity has been detected in blood from healthy individuals and patients with renal and cardiovascular disease (Totsune et al., 2001, 2003; Richards et al., 2002; Russell 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 X1-K2 and X1-R2 bonds would be predicted to cleave between the E11-R12 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.

Materials and 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, preoxygenated (95% O2, 5% CO2) modified Krebs' solution (125 mM NaCl, 5 mM KCl, 2.5 mM CaCl2, 0.5 mM MgSO4, 98.5 mM Cl−, 0.5 mM SO42−, 32 mM HCO3−, 1 mM HPO42−, and 0.04 mM EDTA). Human endothelial cells were cultured from umbilical vein. Umbilical cords were collected in phosphate buffered solution (1.54 mM KH2PO4, 155 mM NaCl, 2.7 mM Na2HPO4·2H2O; 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 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, room temperature), and mesothelial cells were dislodged from the epicardium using a cell scraper. The cell suspension was spun (230g, 6 min, 4°C), the pellet was resuspended in Dulbecco’s modified Eagle’s medium containing 20% fetal bovine serum, penicillin (100 U/ml), and streptomycin (0.1 mg/ml) (20% DMEM solution), and plated into six-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 mm3) were transferred into six-well plates and incubated in 10% DMEM solution. After 2 to 3 weeks, blocks of tissue were removed from the plates and fibroblasts, which had proliferated from the blocks, were collected using trypsin-versene mixture (Cambrex Bio Science Rockland, Inc., Rockland ME). The fibroblast cell suspension was spun (230g, 6 min, 4°C), and the pellet was resuspended in 10% DMEM solution and plated into six-well plates.

Umbilical veins from human umbilical cords were cannulated with a blunt end needle and flushed with DMEM to remove blood. The noncannulated end of the cord was clamped and DMEM containing 1 mg/ml collagenase IV was administered into the vein and incubated for 30 min at room temperature. The cell suspension was collected, spun (230g, 6 min, 4°C), resuspended in 20% DMEM solution, and plated into six-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% CO2 incubator. Culture medium was changed every 3 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; 1:100; 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; DakoCytomation Denmark A/S, Glostrup, Denmark). Fibroblasts stained negatively with a mouse monoclonal antibody to smooth muscle α-actin (clone 1A4; 1:1000; Immunon, Pittsburgh, PA). Immunoreactivity was detected using an 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, Parkville, Australia) was incubated for 3 h at 37°C in incubation medium containing Tris HCl (100 mM; pH 5.0, 7.0, or 8.5), or with or without CaCl2 (1 mM), with or without furin (2 U/ml; New England BioLabs, Beverly, MA), trypsin (0.05 mg/ml, type XI; Sigma-Aldrich, St. Louis, MO), or Triton X-100 (0.1%). Samples containing furin were incubated with or without medium that was depleted of Ca2+ ions and supplemented with 2 mM EDTA, whereas 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 CaCl2 (1 mM) and EDTA (2 mM). Cells were coincubated in the absence (nonpermeabilized cells) or presence of 0.1% Triton X-100 (permeabilized cells) for 3 h 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 (1600g, 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, Taunton, Ireland), which were reequilibrated with 0.1% trifluoro-
acetic 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 of 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 high-performance liquid chromatography (HPLC)-mass spectrometry analysis.

**Liquid Chromatography-Mass Spectrometry.** 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), and then ramped from 100% solvent A to 80% solvent B over 8 min, held at 80% solvent B for 2 min, and 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 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 liquid chromatography-tandem mass spectrometry system. Samples were ionized by positive ion electrospray atmospheric pressure ionization. The ion spray, orifice (O₂) and ring voltages were 5200, 35, and 225 V, respectively. The quadrupole zero (Q₁) voltage was −10 V, and the declustering potential was 45 V (O₂ − Q₁).

An authentic sample of synthetic hU-II (Auspep) was analyzed by liquid chromatography-mass spectrometry, and the retention time of hU-II was found to be 6.7 min (Fig. 2). hU-II was detected as a doubly charged ion ([M + 2H]²⁺). An extracted ion chromatogram ([m/z] 694.4 [M + 2H]²⁺) was generated to define the hU-II peak. The hU-II peak was integrated to allow semiquantitative determination of hU-II (Fig. 3), and the area data were expressed as total ions counted. The sensitivity of detection for hU-II and CTF-ProhU-II was 1 pmol.

**Results**

**Investigation of Furin and Trypsin in the Proteolytic Processing of CTF-ProhU-II.** The proteolytic processing of pro-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; Fig. 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 (Fig. 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 (Fig. 2, B and C). Semiquantitative analysis of hU-II levels in cell-free and biological samples was determined after generation of an extracted ion chromatogram ([m/z] 693–696; Fig. 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 (Fig. 4) and pH 8.5 (Fig. 4, but not at pH 5.0 (Fig. 4). Proteolytic conversion of CTF-ProhU-II was markedly reduced at pH 7.0 when the incubation buffer was depleted of Ca²⁺ ions and supplemented with 2 mM EDTA (Fig. 4). CTF-ProhU-II was also cleaved by trypsin (Fig. 4), and this was sensitive to the trypsin inhibitor aprotinin (Fig. 4).

**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, nonpermeabilized human epicardial mesothelial cells, cardiac fibroblasts, and umbilical vein endothelial cells. Only low levels of mature hU-II were detected in the superfusate of mesothelial cells (Fig. 5), 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 superfusates when cells were incubated with CTF-ProhU-II and permeablized with 0.1% Triton X-100 (Fig. 5). hU-II was not detected when permeablized cells were incubated without CTF-ProhU-II (Fig. 5) and was only weakly detected in a cell-free system in which prohU-II was incubated with 0.1% Triton X-100 (Fig. 5), suggesting that mature peptide was not released from intracellular compartments after cell permeabilization and that the presence of hU-II could not be attributed to spurious cleavage of CTF-ProhU-II by the cell permeabilization 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; Fig. 5), and by the substitution of incubation medium that was depleted of Ca²⁺ ions and supplemented with EDTA (Fig. 5). 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 (Fig. 6). hU-II was not detected in blood incubated without ProhU-II (not
undetected after coincubation with trypsin and 34.5 

ing CTF-ProhU-II, which was incubated with 0.05 mg/ml trypsin, but was 

mented medium. hU-II was readily detected in cell-free medium contain-

tured human epicardial mesothelial cells, vascular endothe-

to investigate urotensin-converting enzyme activity in cul-

activity (Russell et al., 2003), and so the aim of this study was 

Fig. 7B) and in control cells incubated without antibody (Fig. 

Negligible levels of staining were detected over the nucleus 

staining for anti-hU-II peptide was observed in the cytoplasm 

lial cell marker anti-cytokeratin 18 (Fig. 7A). Positive 

sothelial cells were positively identified using the mesothe-

Cultured human epicardial me-
are expressed as total number of ions counted over the period of the hU-II
antibody was negligible (C). Scale bars, 200 μm.

Fig. 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 are expressed as total number of ions counted over the period of the hU-II peak. Values are mean ± S.E.; n = 3; ∗, P < 0.05.

mM) and is inactive in Ca2+-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 (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 it 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 Ca2+ 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 permeabilized 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 permeabilization 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 (Nagayama, 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 enzyme-linked immunosorbent assay in human plasma (Heller et al., 2001; Totsume et al., 2001, 2003; Ng et al., 2002; Richards et al., 2002; Russell 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 (endothelin-1, big endothelin-1; Neri Serneri et al., 2000), adrenomedullin (proadrenomedullin N-terminal 20 peptide; 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 with patients with nonfailing hearts, mature hU-II was not detected using mass spectrometry (Russell et al., 2003), raising the possibility that the antibody recognized 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 prepro-U-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 superfusate of cultured, nonpermeabilized 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 X1-K2 and X1-R2 bonds would be predicted to cleave between the E11-R12 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/l (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 fibro-
blasts 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.

Acknowledgments

We thank the surgeons and theater staff of The Prince Charles Hospital for assistance with collection of cardiac tissue, Anne Carle for assistance in patient recruitment, Anne Carle and Pam Cleave for collection of blood samples, and Lillian Yan and staff of The Royal Women’s Hospital for assistance in the collection of umbilical cords.

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